

Title: Structural Insights into Ligand Recognition, Selectivity and Activation of the human Bombesin Receptor Subtype-3

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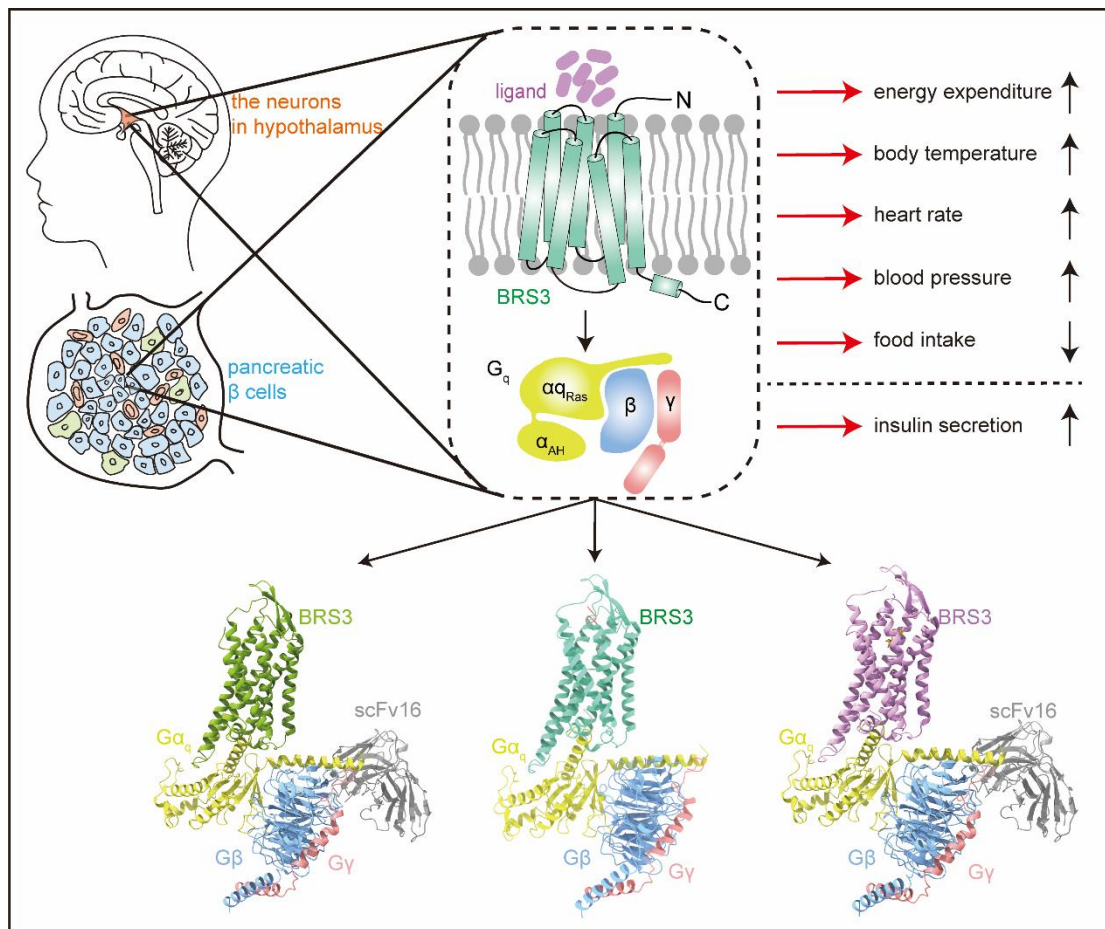
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32 **Graphical Abstract**



34 **In Brief**

35 Molecular basis for recognition of exogenous agonists by the human bombesin
 36 receptor subtype-3 provides avenues for design of new modulators for diabetes and
 37 obesity.

39 **Highlights**

- 40 • Structures of BRS3-G_q complexes, both in the absence (apo-form) and
 41 presence of the ligands BA1 and MK-5046, were determined.
- 42 • The important residues in the ligand binding pocket for bombesin receptor
 43 subtype-3 activation were described.
- 44 • The basis for ligand selectivity of bombesin receptors was depicted in detail.
- 45 • Resolution of the binding pockets provides path for rational drug design against
 46 bombesin receptor subtype-3 for the treatment of diabetes and obesity.

SUMMARY

Bombesin receptor subtype-3 (BRS3) is an important orphan G protein-coupled receptor that regulates energy homeostasis and insulin secretion. As a member of the bombesin receptor (BnR) family, which includes neuromedin B receptor (NMBR) and gastrin-releasing peptide receptor (GRPR), the lack of known endogenous ligands and high-resolution structure has impeded understanding of BRS3 signaling and function. Here, we present cryogenic electron microscopy (cryo-EM) structures of BRS3 in complex with heterotrimeric G_q protein in three states: apo, bound to the pan-BnR agonist, BA1, and bound to the synthetic BRS3-specific agonist MK-5046. These structures reveal the architecture of the orthosteric ligand pocket underpinning molecular recognition. Comparisons with BnR members provide insights into the structural basis for BRS3's selectivity and low affinity for bombesin peptides. Examination of conserved micro-switches suggests a shared activation mechanism among BnRs. Together our results enable deeper exploration of BRS3's ligand selectivity, signaling, and therapeutic targeting for diabetes and obesity.

Introduction

G protein-coupled receptors (GPCRs), pivotal membrane proteins, orchestrate cellular responses to a variety of extracellular stimuli, including peptide ligands. Among these, bombesin, a 14-amino acid peptide originally isolated from the European xenopus laevis (*Bombina bombina*)^{1,2}, stands out. In mammals, bombesin analogs such as neuromedin B (NMB) and gastrin-releasing peptide (GRP) interact with specific receptors in the bombesin receptor (BnR) family³. These receptors are integral to physiological processes in the gastrointestinal tract and central nervous system, influencing aspects like food intake⁴, fear memory consolidation⁵, and itch sensation^{6,7}. Structural studies of NMBR and GRPR with various ligands have shed light on the interactions within the BnR family, enhancing our understanding of their structure-function relationships and aiding in the development of novel therapeutics for BnR-related conditions, including cancers and pruritus^{8,9}.

Bombesin receptor subtype-3 (BRS3), also a member of the BnR family, is an orphan GPCR primarily found in the brain. Unlike its counterparts NMBR and GRPR, BRS3 does not exhibit high affinity for the known endogenous bombesin family peptides^{3,10,11}(Table S1), and its natural ligand remains elusive. Despite this, specific ligands have been identified for BRS3. Among them, the synthetic bombesin analog [D-Phe⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]Bn(6–14) (where D and β indicate Dextro- and β -conformations, respectively), referred to as BA1, exhibits high affinity, binding to all three bombesin family receptors. As a pan-BnR agonist, BA1 is frequently utilized in research as a BRS3 agonist^{12,13}. Nonpeptide agonists like MK-5046 have shown selective BRS3 agonism, enhancing our ability to investigate BRS3's functions^{14,15}. Additional agonists, such as DSO-5a and oridonin, have displayed potent and selective BRS3 activation, suggesting their potential in treating metabolic disorders^{16,17}. Particularly, in comparison with MK-5046, DSO-5a activates peripheral BRS3 to control blood glucose homeostasis while circumventing central effects¹⁸. Additionally, oridonin, derived from *Isodon rubescens* and commonly employed in Traditional Chinese Medicine¹⁷, exhibits pharmacological activities encompassing hypoglycemic and anti-inflammatory effects¹⁹, as well as anti-cancer effects²⁰. As a novel BRS3 ligand, oridonin holds promise as a lead compound for treating metabolic disorders, including type 2 diabetes and obesity.

Utilizing these synthetic ligands and animal models has revealed BRS3's critical roles in regulating energy homeostasis, glucose metabolism, and blood pressure. Its significant function in obesity was initially identified²¹, followed by findings underscoring its necessity for energy balance and insulin secretion²²⁻²⁴. BRS3 neurons in the hypothalamus can be activated by cold exposure, refeeding, and certain agonists. Notably, activation of BRS3 neurons in the dorsomedial hypothalamus increased sympathetic nervous tone, elevating energy expenditure, body temperature, heart rate, and blood pressure via brown adipose tissue, heart, and arteries, respectively, without affecting food intake or physical activity. Conversely, the activation of BRS3 neurons

in the paraventricular nucleus of the hypothalamus reduced food intake, without affecting energy expenditure, body temperature, or physical activity²⁵ (Figure 1A). Additionally, agonists of BRS3 can stimulate insulin secretion from pancreatic β cells (Figure 1A), making BRS3 a promising target for metabolic diseases such as obesity and diabetes²⁶. However, the lack of structural data for BRS3, especially in different ligand-bound states, has hindered our understanding its molecular mechanisms and the development of targeted treatments.

In this study, we present cryo-EM structures of BRS3 in active conformations, both in the absence and presence of ligands (BA1 and MK-5046). Together with mutational studies, these structures elucidate the mechanisms of ligand binding and receptor activation, offering crucial insights into this orphan receptor. This information is invaluable for structure-based drug design targeting BRS3, potentially revolutionizing treatment approaches for obesity, diabetes, hypertension, and related diseases.

Results

Structure determination of BRS3-G_q complexes

For the cryo-EM analysis of BRS3-G_q complexes, we engineered the human BRS3 by attaching a thermostabilized apocytochrome b⁵⁶²RIL (BRIL) to its N-terminus. This was done to enhance the stability and homogeneity of the complex, crucial for effective cryo-EM studies²⁷ (Figure S2). Additionally, we utilized the NanoBiT tethering strategy: fusing the large part of NanoBiT (LgBiT) to the C-terminus of BRS3 and a 13-amino acid NanoBiT peptide (HiBiT) to the C-terminus of the G β subunit²⁸. We also employed an engineered G_q protein chimera, similar to the one used in prior structural studies of NMBR and GRPR⁸. Here, 'G_q' refers to this specific engineered chimera. We assembled the BRS3-G_q complexes for cryo-EM by co-expressing and purifying BRS3-LgBiT, G_q, G β γ -HiBiT, antibody scFv16, and nanobody-35 (Nb35)^{29,30}. These components were processed both in the absence (apo-form) and presence of the ligands BA1 and MK-5046. This approach facilitated the efficient formation and enhanced stability of the apo and ligand-bound BRS3-G_q complexes.

The single-particle cryo-EM analysis of the collected datasets yielded density maps at nominal global resolutions of 3.5, 2.9, and 3.0 Å for the apo BRS3-G_q-scFv16, BA1-bound BRS3-G_q, and MK-5046-bound BRS3-G_q-scFv16-Nb35 complexes, respectively (Figures 1B-1G, S3-5; Table S2). These maps clearly delineated the positions of the receptor, G-protein trimer, and scFv16/Nb35 in each complex, with most amino acid side chains being resolved (Figure S6). In the apo BRS3-G_q-scFv16 complex, the orthosteric binding pocket (OBP) did not exhibit any additional densities. However, for the BA1- and MK-5046-bound complexes, densities for BA1 and MK-5046 were well-resolved, allowing for the construction of atomic models with a high degree of confidence. All three complexes displayed a similar overall structural arrangement, with root mean square deviation (RMSD) values for Cα atoms ranging between 0.3-0.6 Å, indicating minor variations among them (Figures S7A-S7C).

Recognition of BA1 by BRS3

In our study, the quality of the cryo-EM maps allowed for the precise modeling of all nine amino acid residues of BA1. This finding aligns with our previous structures of NMB-NMBR and GRP(14-27)-GRPR, where only the C-terminal 9 residues of the longer bombesin agonists (33-residue peptide NMB30 and 14-residue peptide GRP(14-27), respectively) were observed, despite the utilization of the 33-residue peptide NMB30 and the 14-residue peptide GRP(14-27) during complex preparation, respectively (Figures 2A and 2B; Table S3). This suggests that a minimal 9-residue segment might be sufficient for full agonist potency. In the BRS3 structure, BA1 is positioned within the central OBP located in the upper transmembrane domain (TMD), interacting with all extracellular loops and transmembrane segments except TM1. The peptide's C-terminus penetrates deeply into the helical bundle, while the N-terminus of BA1 extends outward towards the extracellular ligand binding cavity. BA1 assumes a dumbbell-shaped conformation, reminiscent of NMB and GRP(14-27) in their respective complexes, underlining a conserved recognition pattern among bombesin

receptor family agonists (Figure 2B). For clarity, all positions of BA1 are numerically designated from the amino terminus of NMB or NMC (Figure 2A).

The nine residues of BA1 (residues 2-10) can be categorized into three motifs: the C-terminal HFX(8-10)^{BA1} motif (Where the X is Nle), the middle AV^BA(5-7)^B motif, and the N-terminal ^DFQW(2-4)^{BA1} motif (Figure 2A). The HFX(8-10)^{BA1} motif adopts an extended structure at the bottom of the OBP, with the side chain of X10^{BA1} penetrating the deepest. The side chains of X10^{BA1} and F9^{BA1} are encased in a hydrophobic cavity comprising L96^{2.53}, L128^{3.33}, V131^{3.36}, C221^{5.42}, F225^{5.46}, W284^{6.48}, A319^{7.42}, and F320^{7.43} (Figure 2C). These hydrophobic interactions are crucial for BRS3 activation, as demonstrated by significantly reduced potency when L96^{2.53}, L128^{3.33}, and V131^{3.36} are substituted with alanine (Figures 2F and S8; Table S4). In addition, F9^{BA1} engages in stabilizing π - π packing interactions with the surrounding F225^{5.46}, H288^{6.53} and Y291^{6.55} (Figure 2D). The H8^{BA1} residue forms hydrogen bonds with S124^{3.29} and S205^{ECL2}, differing from the sandwiching of the corresponding H8 residues in NMB and GRP(14-27) by P^{3.29} and P^{ECL2} in the NMB-NMBR and GRP(14-27)-GRPR structures (Figures 2D and S7F). The alanine mutations of S124^{3.29} or S205^{ECL2} had a minimal effect on BRS3 activation by BA1, yet, the proline substitution of the S124^{3.29} or S205^{ECL2} substantially lowered the BRS3 activity coordinated by BA1 (Figures 2D and S8; Table S4). This highlights the pivotal roles of S124^{3.29} and S205^{ECL2} in peptide BA1 recognition by BRS3. H8^B also forms hydrogen bonds with R127^{3.32} and E182^{4.60} (Figure 2D). Furthermore, the R127^{3.32} also forms a hydrogen bond with the backbone carbonyl of the F9^B, and the R316^{7.39} forms a hydrogen bond with the backbone carbonyl of the H8^B (Figure 2D). The carboxyl group of the X10^{BA1} forms two hydrogen bonds with the side chains of the R127^{3.32} and R316^{7.39} (Figure 2D). Mutation of R127^{3.32}, E182^{4.60}, C221^{5.42}, H288^{6.52}, Y291^{6.55} and R316^{7.39} in BRS3 results in a reduced BA1-induced signaling response (Figures 2F, 2G and S8; Table S4), confirming the essential roles of these residues in BA1 binding and receptor activation.

The AV^βA(5-7)^{BA1} motif primarily engages in hydrophobic interactions with F191^{ECL2} and F200^{ECL2} from the ECL2, and P304^{ECL3} from ECL3 (Figure 2D). Indeed, alanine mutation of the F200^{ECL2} resulted in decrease in G_q signaling (Figures 2G and S8; Table S4). In addition to the hydrophobic interactions, the backbone carbonyl group of ^βA7^{BA1} formed a hydrogen bond with the backbone amide group of S205^{ECL2}, further stabilizing BA1 binding.

Following the middle motif, the ^DFQW(2-4)^{BA1} motif extends along the upper pocket, engaging in π-π interactions with Y108^{2.65}, F200^{ECL2}, H308^{7.31}, and F309^{7.32} from BRS3 (Figure 2E). Alanine substitutions at these sites diminish receptor activation compared to the wild-type BRS3 (Figure 2G; Table S4). Anion-π interactions between ^DF2^{BA1} and D193^{ECL2}/D303^{ECL3} also contribute to BA1's binding stability, with alanine mutation of D303^{ECL3} significantly reducing G_q signaling (Figure 2G; Table S4). Furthermore, Q3^{BA1} forms extensive hydrogen bonds with the BRS3 backbone, and its carbonyl group interacts with H107^{2.64} (Figure 2E). In summary, these intricate details reveal the comprehensive mechanism behind BRS3 activation by the synthetic peptide BA1, highlighting the specific interactions and critical residues involved in this process.

BRS3 regulation by MK-5046

Continuing our exploration of BRS3, we examined its interaction with MK-5046, the first BRS3-specific nonpeptide receptor agonist (Figure 3). MK-5046 is characterized by distinct functional groups, including a hydroxyl group and a trifluoromethyl group in the central segment, an additional trifluoromethyl group accompanied by a cyclopropane group and an imidazole group on one side, and a benzene ring and a pyrazole group on the other (Figure 3B). The structure of MK-5046-bound BRS3 reveals that MK-5046 adopts a "V"-shaped conformation, fitting snugly at the bottom of the BRS3 pocket. Notably, MK-5046, being smaller in volume (475 Å³) than BA1 (1089 Å³), occupies a specific sub-pocket that corresponds to the C-terminal HFX(8-10)^{BA1} motif (Figure 3A).

The trifluoromethyl group, connected to the cyclopropane group, projects towards the upper portion of TM3, establishing extensive fluorine-based halogen bonding interactions with S124^{3.29}, R127^{3.32}, and E182^{4.60} (Figure 3C). Mutations in R127^{3.32} and E182^{4.60} lead to significantly reduced MK-5046-induced signaling (Figures 3E and S8; Table S4), emphasizing the critical roles of these residues in MK-5046 binding and BRS3 activation. Replacement of R127^{3.32} with Q^{3.32} in both NMBR and GRPR receptors notably decreased the E_{max} level for mutated BRS3 (Figures 3E and S8; Table S4), underlining R127^{3.32}'s significance in MK-5046's selectivity for BRS3.

Additionally, the imidazole, benzene, and pyrazole rings of MK-5046 engage in extensive π - π interactions with W284^{6.48} and Y291^{6.55} of BRS3 (Figure 3C). The interaction between MK-5046's imidazole group and Y291^{6.55} is further reinforced by a hydrogen bond with S205^{ECL2} (Figure 3C). Mutating S205^{ECL2} and Y291^{6.55} results in a considerable decrease in MK-5046-induced BRS3 signaling (Figures 3E and S8; Table S4), confirming their essential role in this interaction. The central hydroxyl group of MK-5046 forms a hydrogen bond with C221^{5.42}, while the central trifluoromethyl group creates robust packing interactions with H288^{6.52}, complemented by π - π packing between W284^{6.48} and H288^{6.52} (Figures 3C and 3D). The opposite side of MK-5046, featuring a benzene ring and pyrazole group, is accommodated within a hydrophobic pocket formed by L96^{2.53}, V131^{3.36}, F225^{5.46}, W284^{6.48}, A319^{7.42}, and F320^{7.43} (Figure 3D).

Previous competitive assays involving MK-5046, Peptide-1 (another pan-BnR agonist), and Bantag-1 (a BRS3-specific peptide antagonist) suggested that MK-5046 functions as an allosteric agonist for BRS3, possibly binding to a distinct pocket from Peptide-1³¹. Our structures show that MK-5046's binding site and pose closely resemble the C-terminal HFX(8-10)^{BA1} motif of BA1 (Figure 3B). The upper trifluoromethyl group of MK-5046 corresponds to the binding site for BA1's H8^B, while its central trifluoromethyl

group and benzene ring align with the lower hydrophobic cavity, mirroring the FX(9-10)^B side chains of BA1. The pyrazole group of MK-5046 assumes a position akin to the carboxyl group of BA1's X10^B. In summary, MK-5046's binding pattern shares similarities with the C-terminal HFX(8-10)^{BA1} tripeptide of BA1, suggesting that MK-5046 is an orthosteric agonist. Interestingly, alanine substitution of R316^{7,39} enhances BRS3 activity induced by MK-5046, showing higher efficacy in our IP1 assay (Figure 3F and S8; Table S4). This finding further underscores the intricate molecular interactions that govern BRS3 regulation and activation by MK-5046.

Activation of BRS3 by agonists of different types

Understanding how receptor occupation correlates with activation, particularly in the context of peptide and nonpeptide agonists for GPCRs, is crucial. Interestingly, BRS3 exhibits a unique behavior in this regard. The binding affinities of the peptide agonist BA1 and the nonpeptide agonist MK-5046 are 2.0 nM and 17 nM^{10,32}, respectively, indicating that BA1 has a nearly 10-fold higher affinity for the receptor than MK-5046. Correspondingly, analysis using dr_sasa reveals that the interface areas for BA1 and MK-5046 are 1094.26 Å² and 429.02 Å², respectively³³. This suggests a direct relationship between a larger buried surface area in the structure and a higher binding affinity.

However, the activation potency of these agonists presents an intriguing contrast. In our IP1 assays, MK-5046 activates BRS3 with an EC₅₀ value of 0.5 nM, demonstrating 26-fold greater potency in activating G_q signaling than BA1, which has an EC₅₀ of 13.0 nM. This is notable as most nonpeptide agonists typically exhibit lower potency than the natural peptide agonists of their respective receptors in activating downstream signaling. Yet, MK-5046 surpasses the peptide ligand BA1 in terms of G_q signaling activation.

To further investigate the potency differences between MK-5046-BRS3 and BA1-BRS3,

we conducted molecular dynamics (MD) simulations, each spanning 500 ns over three replicates. These simulations focused on the ligand-binding domain composed of TM3, TM5, and TM6, a region known to influence ligand potency³⁴. The simulations revealed distinct hydrophobic interaction networks in the extracellular TM3-TM5-TM6 region for the two agonists. Specifically, in the MK-5046-BRS3 system, a hydrophobic network surrounding the trifluoromethyl group of MK-5046 was present in 62.9% of the trajectories, compared to only 23.7% in the BA1 system around F9^{BA1}. Representative structures (Figure 4A) showed that the trifluoromethyl group in MK-5046 engages in electronic interactions with H288^{6.52} and facilitates hydrophobic interactions between F225^{5.46} and V131^{3.36}. In contrast, the larger F9^{BA1} moiety in the BA1 system interacts primarily with V131^{3.36}, disrupting the hydrophobic contact between F225^{5.46} and V131^{3.36} (Figure 4B).

Moreover, in the MK-5046 system, the trifluoromethyl group acts as a hydrophobic bridge between H288^{6.52} and F225^{5.46}, stabilizing the activated extracellular TM3-TM5-TM6 conformation. This is evidenced by the fact that direct contact between H288^{6.52} and F225^{5.46} is less frequent (19.9% versus 78.1% in the BA1 system). Consistent with this, the H288A mutation significantly diminishes MK-5046's potency (from 0.53 nM to 126.9 nM), while having a less pronounced effect on BA1's binding (from 11.8 nM to 68.7 nM) (Figures 2G, 3F, and 4C). In summary, these findings illustrate the complex and distinct mechanisms by which different types of agonists, both peptide and nonpeptide, activate BRS3. They also highlight the nuanced molecular interactions that govern the receptor's response to these agonists, contributing to our understanding of GPCR activation and signaling.

Basis for ligands selectivity of bombesin receptors

In the BnR family, BA1, a synthetic analog, was found to have a high affinity for BRS3, as well as for NMBR and GRPR receptors^{10,35,36} (Table S1). BRS3 shares approximately 50% amino acid sequence identity with NMBR and 47% with

GRPR^{11,37,38}. A closer examination of BA1 binding in BRS3 and GRPR indicates that this synthetic peptide uniquely coordinates with all three bombesin receptors. Structural alignment of the BA1-GRPR-G_q complex (PDB: 7W40)⁹ with our BA1-BRS3-G_q structure showed a high degree of similarity, with an overall C α RMSD of 0.75 Å (Figure 5A). Both the ^DFW(2-4)^B and HFX(8-10)^{BA1} motifs of BA1 align well between BRS3 and GRPR, with notable differences in the side chains of ^DF2^{BA1}, W4^{BA1}, and H8^{BA1} (Figure 5B). The key residues in BRS3's upper pocket, including Y108^{2,65}, F200^{ECL2}, H308^{7,31}, and F309^{7,32}, involved in π - π interactions with ^DF2^{BA1} and W4^{BA1}, are highly conserved in GRPR without substitutions (Figure 5C), suggesting a preserved interaction pattern despite conformational differences in these aromatic amino acids. However, in BRS3, the H8^{BA1} side chain deviates from TM3, causing a shift of the middle AV ^{β} A(5-7)^{BA1} motif towards TM6, compared to its position in GRPR (Figure 5D). This shift is stabilized by two hydrogen bonds between H8^{BA1} and S124^{3,29} and S205^{45,52} in BRS3. In contrast, BA1's H8 is sandwiched by P^{3,29} and P^{45,52} in GRPR, resembling the coordination seen in NMB-NMBR and GRP(14-27)-GRPR structures (Figure S7F). The significant sequence homology and specific flexibility, especially in the ligand-binding pockets, explain BA1's ability to bind all three bombesin receptors.

The selectivity of natural bombesin-related peptide agonists for individual bombesin-related receptors is an interesting aspect. GRP, the natural ligand for GRPR, has an affinity for GRPR over 650-fold higher than for NMBR and more than 15,000-fold higher than for BRS3. NMB, the natural ligand for NMBR, shows over 650-fold higher affinity for NMBR compared to GRPR and more than 20,000-fold higher than for BRS3 (Table S1). Prior studies have clarified the determinants of selectivity between NMB30 and GRP(14–27) for NMBR and GRPR^{8,39,40}. In our sequence and structure analyses of these receptors bound with different peptides, we observed five amino acids in the peptide agonist binding pockets that are conserved in both GRPR and NMBR but differ in BRS3: T106^{2,63}, H107^{2,64}, R127^{3,32}, S205^{ECL2}, and H294^{6,58} in BRS3 are substituted by S^{2,63}, R^{2,64}, Q^{3,32}, P^{ECL2}, and R^{6,58}, respectively (Figure S1), in NMBR and GRPR. A

structure comparison of BA1-BRS3 with NMB-NMBR shows a clash between T6^{NMB} of NMB and S202^{ECL2} of BRS3, which is absent in the equivalent interaction in the BRS3 structure due to a shift of BA1's middle motif towards TM6 (Figure 5E). In the BRS3 structure, the equivalent V6^{BA1} residue of BA1 shift away from the S202^{ECL2}, due to the shift of the middle AV^βA(5-7)^{BA1} motif towards the TM6. This shift of BA1 middle motif may be a structural characteristic of BRS3 specific ligand, which is favored by the residue combination of the R127^{3.32} and H294^{6.58} substitutions in BRS3 (Figure 5E). Through structural analysis, it is evident that BA1 presents a binding pose of the middle motif nearly identical to the natural agonist GRP in the GRPR structures^{8,9}, located close to TM6 (Figure 5F). Interestingly, the NMB middle motif exhibits a closer alignment with TM2 (Figure 5F). Consequently, these ligand binding poses of NMB and GRP are favored by the conserved residue combination of the Q^{3.32} and R^{6.58} substitutions in NMBR and GRPR receptors. Thus, the Q^{3.32} and R^{6.58} substitutions contribute to the structural characteristics of ligand specific for individual Bn-related receptors, as suggested in the prior mutation studies⁴¹. For NMB, it has been reported that T6^{NMB} forms a hydrogen bond with the main carboxyl group of L3^{NMB}, playing a pivotal role in NMB binding and selectivity by NMBR^{8,9}. The clash of the T6^{NMB} here possibly contributes to the weak binding of the NMB by BRS3.

In addition, structure comparison of the BA1-BRS3 with our previously reported GRP(14-27)-GRPR structure (PDB code: 8H0Q) reveals that the T106^{2.63} substitution in BRS3 induces an inward push of the side chain of W113^{ECL1} (Figure 5G). This movement is further stabilized by the packing between the side chain of W113^{ECL1} and the H107^{2.64} substitution in BRS3, along with the conserved disulfide bond formed by C120^{3.25} and C203^{ECL2} (Figure 5G). Together with the main chain carboxyl group of E201^{ECL2}, the inward shift of W113^{ECL1} subsequently results in an inward movement of the EGW(111-113)^{ECL1} of BRS3, causing a clash with the crucial residue H3^{GRP} of GPR(14-27) (Figure 5H). When we compare the BA1-BRS3 with the other GRP-GRPR structure (PDB code: 7W3Z), a clash between the H3^{GRP} and the carboxyl group of

E201^{ECL2} can also be observed (Figure S7G), despite the H3^{GRP} here adopt a rotamer conformation due to the similar ECL1 positions between the BRS3 and GRPR structures. Interestingly, the H3^{GRP} was also reported to accommodate the featured ECL2 of GRPR, and contribute to the weak binding of GRP to NMBR^{8,39}, suggesting its pivotal role of selectivity for GPR(14-27) in BnR family. Overall, these structural insights highlight the unique features of BRS3 that contribute to its low affinity for bombesin-related natural ligands like NMB and GRP.

Common activation features of bombesin receptors subfamily

In examining the significant structural similarities between the BA1- and MK-5046-bound BRS3 complexes, we chose the BA1-BRS3 complex for a detailed analysis of BRS3 activation. Comparing the active BA1-bound BRS3-G_q complex with both the active structure of GRPR bound to BA1 (PDB: 7W40) and the inactive structure of GRPR bound to a synthetic antagonist, PD176252 (PDB: 7W41), we observe that BRS3 exhibits classic activation features typical of Class A GPCRs in response to BA1. Notably, the cytoplasmic end of TM6 in BRS3 displays a pronounced outward movement while the cytoplasmic part of TM7 shifts inward. This rearrangement accommodates the C-terminal $\alpha 5$ helix of the G α_q subunit, a key feature of Class A GPCR activation (Figures 6A-6C). Additionally, the extracellular end of TM1 in the BA1-bound BRS3 shifts 5.3 Å towards TM7, similar to movements observed in NMBR and GRPR structures (Figures 6B and S7H).

In comparison to PD176252, BA1 binds deeper within the orthosteric pocket, surrounded by TM3, TM6, and TM7, and forms hydrophobic contacts with W284^{6.48} (Figure 6D). BA1's binding causes a significant deflection of the W284^{6.48} indole ring, initiating a cascade of conformational changes linked to receptor activation⁴² (Figure 6D). This process mirrors the classic activation mechanism seen in Class A GPCRs, where the displacement of W284^{6.48} exerts a downward force on F280^{6.44} in the conserved P^{5.50}V^{3.40}F^{6.44} core triad, leading to changes in the D^{3.49}R^{3.50}Y^{3.51} motif

(Figures 6D-6F). Additionally, the conserved N^{7.49}P^{7.50}xxY^{7.53} motif is replaced with a N^{7.49}P^{7.50}xxL^{7.53} motif in BRS3. In the structure, BA1's X10^{BA1} carboxyl group forms a hydrogen bond with R316^{7.39}, contributing to activating the N^{7.49}P^{7.50}xxL^{7.53} motif in BRS3. Typically, the conserved Y^{7.53} in this motif undergoes a significant rotation towards TM3, leading to TM7's inward movement⁴³. However, in BRS3, the leucine substitution at Y^{7.53} undergoes a lateral shift towards TM1 and TM2, highlighting a unique yet conserved activation mechanism shared among bombesin receptors.

During BRS3 activation, the P^{5.50}V^{3.40}F^{6.44} and N^{7.49}P^{7.50}xxL^{7.53} motifs, which link the ligand-binding pocket to the G protein-coupling interface, undergo rearrangements. These involve movements of F^{6.44} and L^{7.53}, triggering significant outward shifts of TM5 and TM6 (3.4 Å at R^{5.63}; 8.1 Å at R^{6.30}), thereby facilitating the engagement of the Gαq subunit's C-terminus. This feature is consistent with activation mechanisms observed across GPCRs (Figure 6C; Table S5). These structural insights from our BRS3 studies enhance our understanding of the activation mechanisms within the bombesin receptor subfamily, demonstrating how specific molecular changes lead to receptor activation.

Comparison with the AlphaFold2-predicted structure

We compared our cryo-EM resolved structures with the structure predicted by AlphaFold2, specifically focusing on the receptor domain (Figure S7I). Given the notable structural similarities between the BA1- and MK-5046-bound BRS3 complexes, we selected the BA1-BRS3 complex for further analysis. The resolved BA1-BRS3 structure and the AlphaFold2-predicted structure exhibited somewhat dissimilar overall arrangements, with a Cα RMSD of 1.5 Å. Despite AlphaFold2 capturing the general backbone contours of the receptor, substantial discrepancies existed between the predicted models and experimental structures regarding the assembly of the extracellular and transmembrane domains. Compared to the BA1-BRS3 structure, the AlphaFold2 structure represents an inactive conformation, with the intracellular part of TM5 tilting outward and the intracellular part of TM6 shifting inward. Specifically, the

key residues in the OBP in the AlphaFold2 BRS3 structure, including H107^{2,64}, S124^{3,29}, R127^{3,32}, W284^{6,48} (Figure S7J), adopt distinct positions that cannot further favor the BA1 binding. In conclusion, while the AlphaFold2-predicted model serves as a valuable reference point, obtaining additional empirical structural information is imperative to accurately guide drug design.

Discussion

Our study on BRS3, an orphan member of the bombesin receptor subfamily, offers significant insights into the complex dynamics and mechanisms of GPCRs. As orphan receptors, entities like BRS3, without identified endogenous ligands, present challenges in understanding their roles, particularly in physiological and pathological contexts like metabolic diseases and cancer⁴⁴⁻⁴⁶.

The structural analysis of BRS3 in its apo form and when bound to the synthetic analog BA1 and the nonpeptide agonist MK-5046 reveals a high degree of structural conservation (Figure S7A). This similarity, despite the evolutionary loss of binding to natural ligands like NMB and GRP in placental mammals, underscores the evolutionary adaptability of BRS3⁴⁷. Unlike other orphan receptors that drive constitutive activation through their ECL2s, such as GPR12 and GPR21⁴⁸⁻⁵⁰, BRS3 does not exhibit this feature, indicating unique structural and functional characteristics.

Our investigation reveals unique aspects of ligand recognition and activation in BRS3. The complex interplay of residues within the receptor's binding site, including those contributing to agonist-induced activation, highlights the receptor's intricate regulation mechanisms. These findings provide a foundation for the future exploration of BRS3 as a potential therapeutic target, particularly in diseases linked to dysfunctional GPCR signaling.

In the bombesin receptor family, the synthetic analog BA1 exhibits high affinity for BRS3, NMBR and GRPR. Our structural analysis comparing the BA1 complexes with

BRS3 and GRPR shows that the overall binding mode of BA1 to the two receptors is highly similar despite some differences in their amino acid side chains. This is largely attributable to the high homology of key residues in the ligand binding pockets of these receptors. These findings explain BA1's ability to bind all members of the BnR family. On the other hand, natural peptides like NMB and GRP demonstrate high selectivity for individual BnR receptors. Our sequence and structural comparisons of apo and peptide-bound BRS3 identified five key residues that differ from NMBR and GRPR, which may account for its low affinity for NMB and GRP. This provides evidence to understand the loss of binding to these natural peptides by BRS3. Our study offers insights into the structural basis of ligand selectivity, aiding efforts to design BRS3 peptide ligands with high selectivity.

In addition to these findings, our research aligns with the classical activation features observed in Class A GPCRs, including the outward movement of TM6 and the inward shift of TM7 in response to BA1 binding. This conformational change, particularly the deflection of the W284^{6,48} indole ring, echoes activation mechanisms seen in other GPCRs. Such structural insights into BRS3, in tandem with its evolutionary trajectory, contribute to a deeper understanding of its functions and potential therapeutic implications.

Our comprehensive study, therefore, not only unravels the unique structural and functional attributes of BRS3 but also enhances the overall understanding of GPCR activation mechanisms. This knowledge is crucial for developing targeted interventions for diseases associated with GPCR dysfunction. Future research is needed to delve deeper into BRS3's self-activation mechanism and explore its potential as a therapeutic target within the bombesin receptor family.

Competing interests

The authors declare no competing interests.

Data Resources

Materials are available from the corresponding authors upon reasonable request. Density maps and structure coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (PDB) with accession codes EMD-38927 and 8Y51 for apo BRS3-G_q-scFv16 complex; EMD-38928 and 8Y52 for BA1-bound BRS3-G_q complex; and EMD-38929 and 8Y53 for MK-5046-bound BRS3-G_q complex. Source data are provided with this paper.

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Figure 1

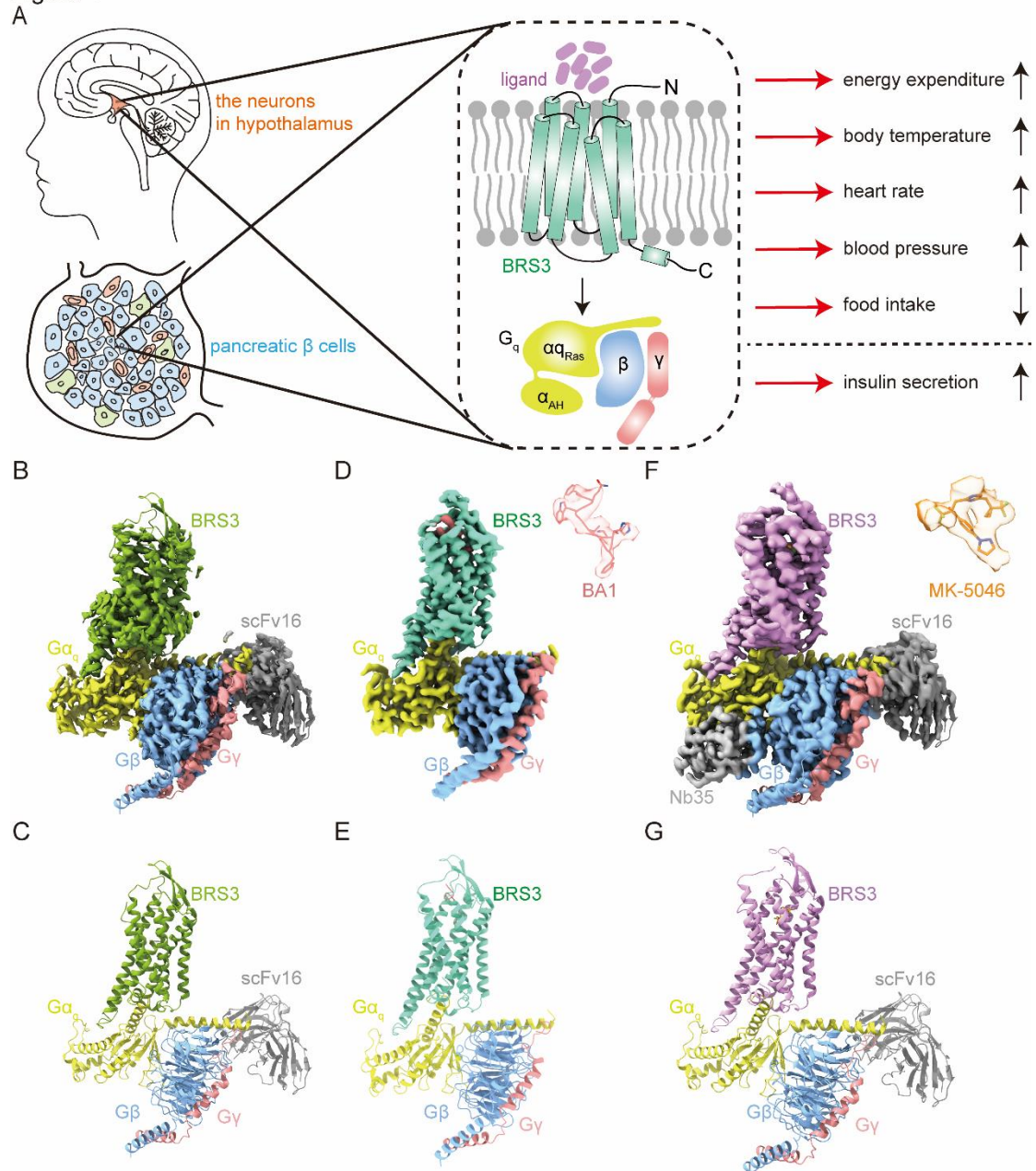


Figure 1. Cryo-EM structures of BRS3- G_q complexes, both in the absence (apo-form) and presence of the ligands BA1 and MK-5046.

(A) Schematic illustration of the BRS3 function in the regulation of metabolism pathways. Briefly, activation of BRS3 neurons in the hypothalamus increased sympathetic nervous tone, elevating energy expenditure, body temperature, heart rate, and blood pressure via brown adipose tissue, heart, and arteries, respectively. And agonists activate BRS3 on pancreatic β cells, leading to an increase in glucose-

stimulated insulin secretion.

(B and C) Orthogonal views of the density map (B) and model (C) for the apo BRS3-
G_q complex. The receptor BRS3 is displayed in forest green. The heterotrimeric G_q
proteins are colored by subunits: Gα_q, yellow; Gβ, cornflower blue; Gγ, deep salmon;
scFv16, gray.

(D and E) Orthogonal views of the density map (D) and model (E) of the BA1-BRS3-
G_q complex. BA1 is shown in light salmon; the receptor BRS3 is displayed in
aquamarine. The heterotrimeric G_q proteins are colored by subunits: Gα_q, yellow; Gβ,
cornflower blue; Gγ, deep salmon.

(F and G) Orthogonal views of the density map (F) and model (G) of the MK-5046-
BRS3-G_q complex. MK-5046 is shown in orange; the receptor BRS3 is displayed in
violet. The heterotrimeric G_q proteins are colored by subunits: Gα_q, yellow; Gβ,
cornflower blue; Gγ, deep salmon; scFv16, gray.

Figure 2

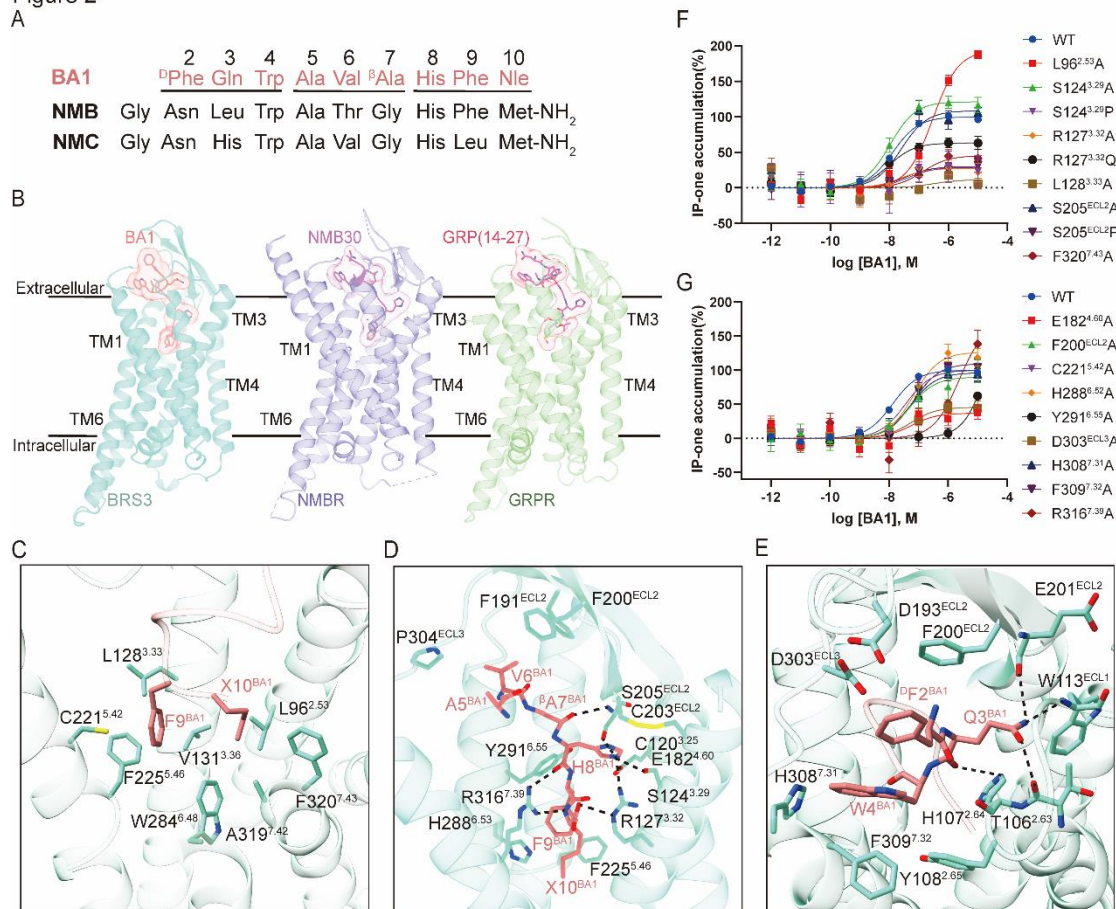


Figure 2. The BA1-binding pocket in BRS3.

(A) The sequence of BA1 peptide. The amino acid numbering of BA1 is determined relative to NMB. See also Table S3.

(B) Side views of the ligands-binding pocket in bombesin receptors family.

(C-E) Detailed interactions of BA1 with BRS3. Hydrogen bonds are depicted as black dashed lines.

(F and G) Effects of mutations in the BA1-binding pocket in IP1 assay. Data are presented as mean \pm S.E.M. of at least three independent experiments. Source data are available online.

Figure 3

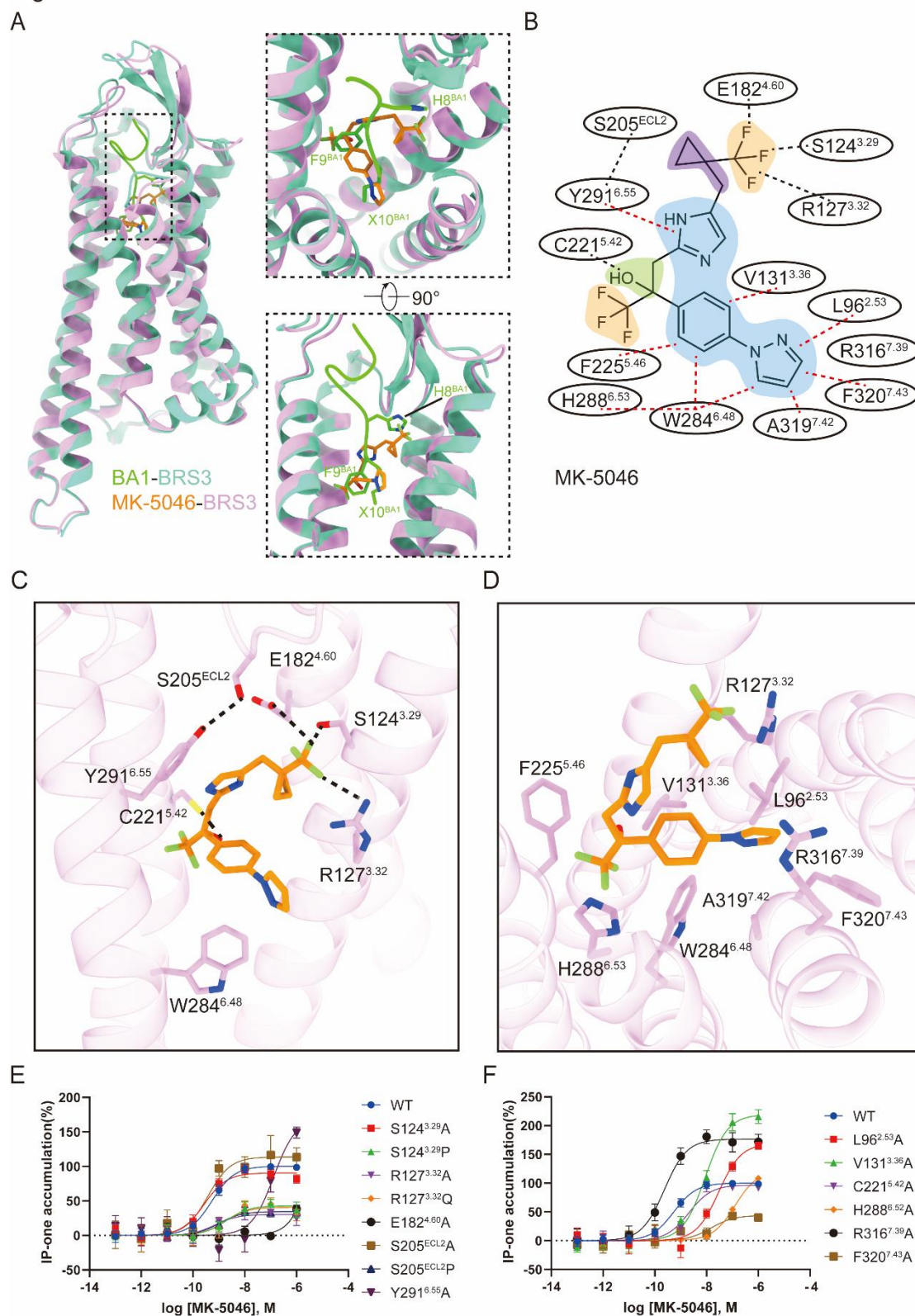


Figure 3. The MK-5046-binding pocket in BRS3.

(A) The chemical structure of MK-5046 and detailed interactions between MK-5046 and BRS3.

(B) The structural comparison of BA1-BRS3 and MK-5046-BRS3 complexes. The binding site and pose of MK-5046 closely resemble the C-terminal dumbbell end HFX(8-10)^{BA1} motif of BA1.

(C and D) Detailed interactions of MK-5046 with BRS3. Hydrogen bonds are depicted as black dashed lines.

(E and F) Effects of mutations in the MK-5046-binding pocket in IP1 assay. Data are presented as mean \pm S.E.M. of at least three independent experiments. Source data are available online.

Figure 4

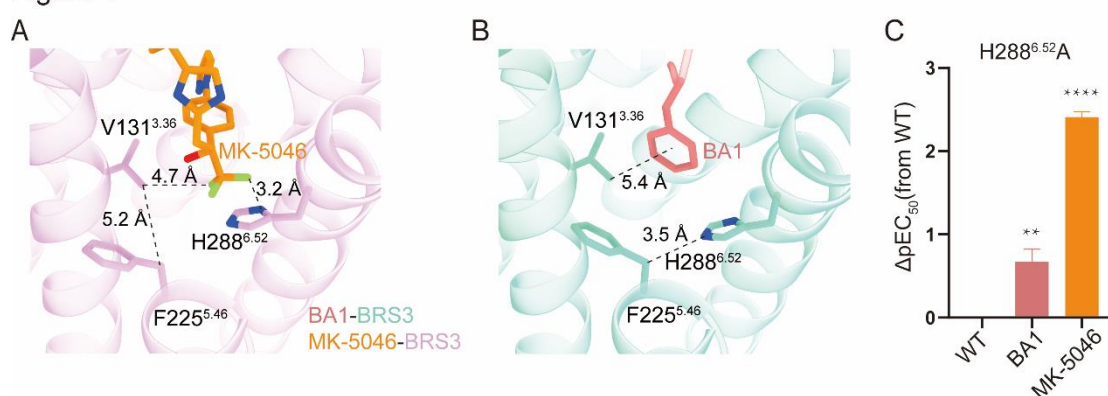


Figure 4. Activation of BRS3 by agonists of different types

(A and B) The representative structures revealed the difference in the MK-5046-BRS3 (A) and BA1-BRS3 (B) complexes.

(C) Effects of H288^{6.52}A on the potency in IP-1 assay. Data were shown as ΔpEC₅₀ ± S.E.M. from three independent experiments, which performed in triplicates, with total repeats of nine for each data point. Statistical differences between wild type (WT) and mutants were determined by two-sided, one-way analysis of variance (ANOVA) with Tukey test. *, P < 0.05; **, P < 0.01; ****, P < 0.001; ns, no significant difference.

Figure 5

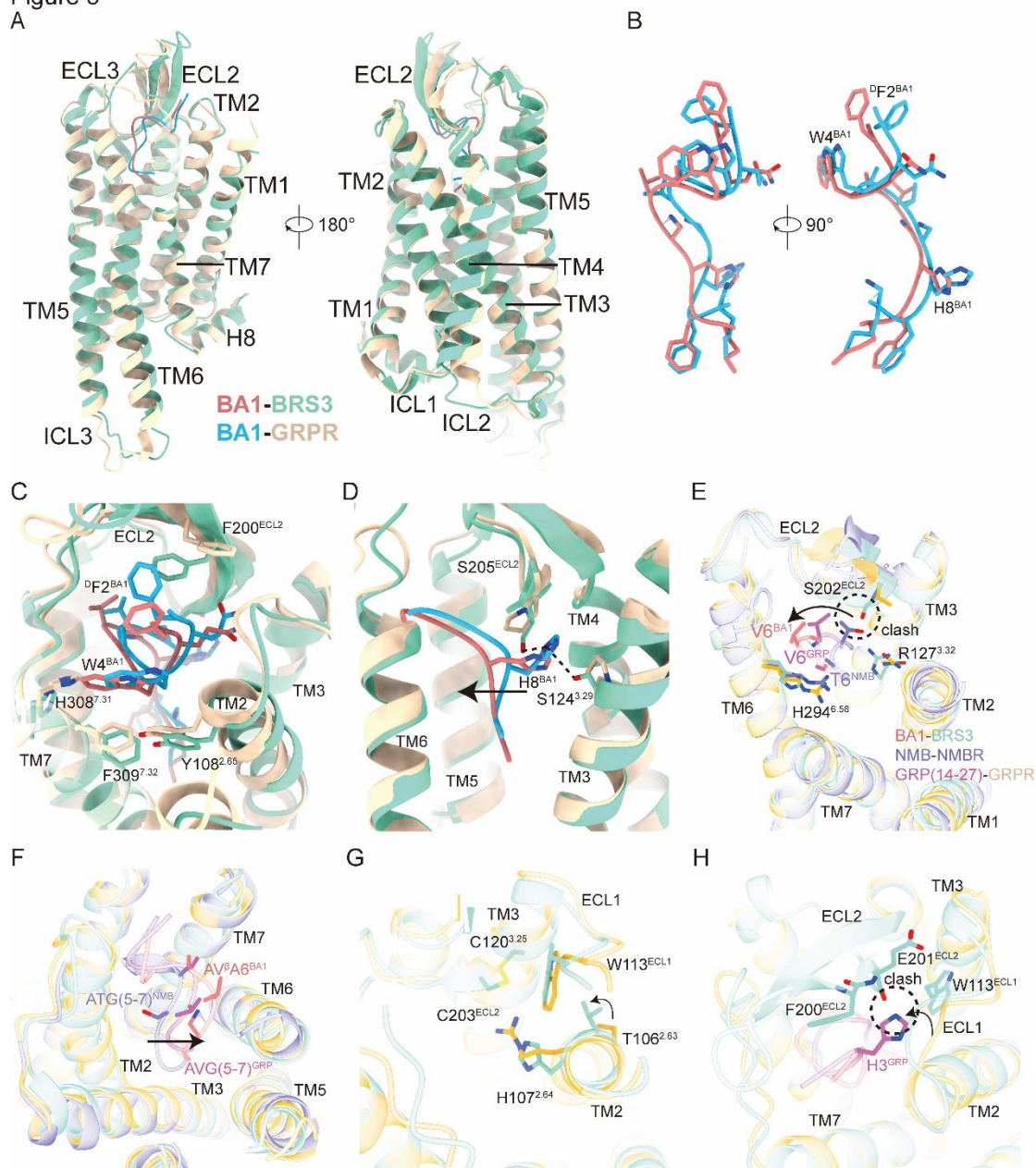


Figure 5. Molecular basis of ligands selectivity of bombesin receptors.

(A) A structural comparison of BA1-BRS3 and BA1-GRPR complexes. The BA1 in BA1-BRS3 complex is shown in light salmon; the receptor BRS3 is displayed in aquamarine; the BA1 in BA1-GRPR complex is shown in dodger blue; the receptor GRPR is displayed in burly wood.

(B) A structural comparison of BA1 conformation in BA1-BRS3 and BA1-GRPR complexes.

(C and D) Detailed interactions between $^{DF2}BA1$, $^{W4}BA1$ (C), and $^{H8}BA1$ (D) with residues in GRPR and BRS3. The shift of middle $^{AV^BA(5-7)}BA1$ motif is represented

818 by an arrow.

819 (E) Detailed interactions between T6^{NMB}, V6^{GRP}, and V6^{BA1} with residues in NMBR,
820 GRPR and BRS3. The shift of T6^{NMB} is represented by an arrow.

821 (F) Detailed interactions between L3^{NMB}, H3^{GRP}, and Q3^{BA1} with residues in NMBR,
822 GRPR and BRS3. The middle motif of BA1 and GRP exhibits a closer alignment with
823 TM6, and the counterpart of NMB closed to TM2.

824 (G) The inward push of the side chain of W113^{ECL1} by T106^{2.63} substitution and the
825 packing between the side chain of W113^{ECL1} and the H107^{2.64} substitution in BRS3.

826 (H) The clash between H3^{GRP} of GPR(14-27) and EGW(111-113)^{ECL1} of BRS3 is
827 represented by a circle.

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Figure 6

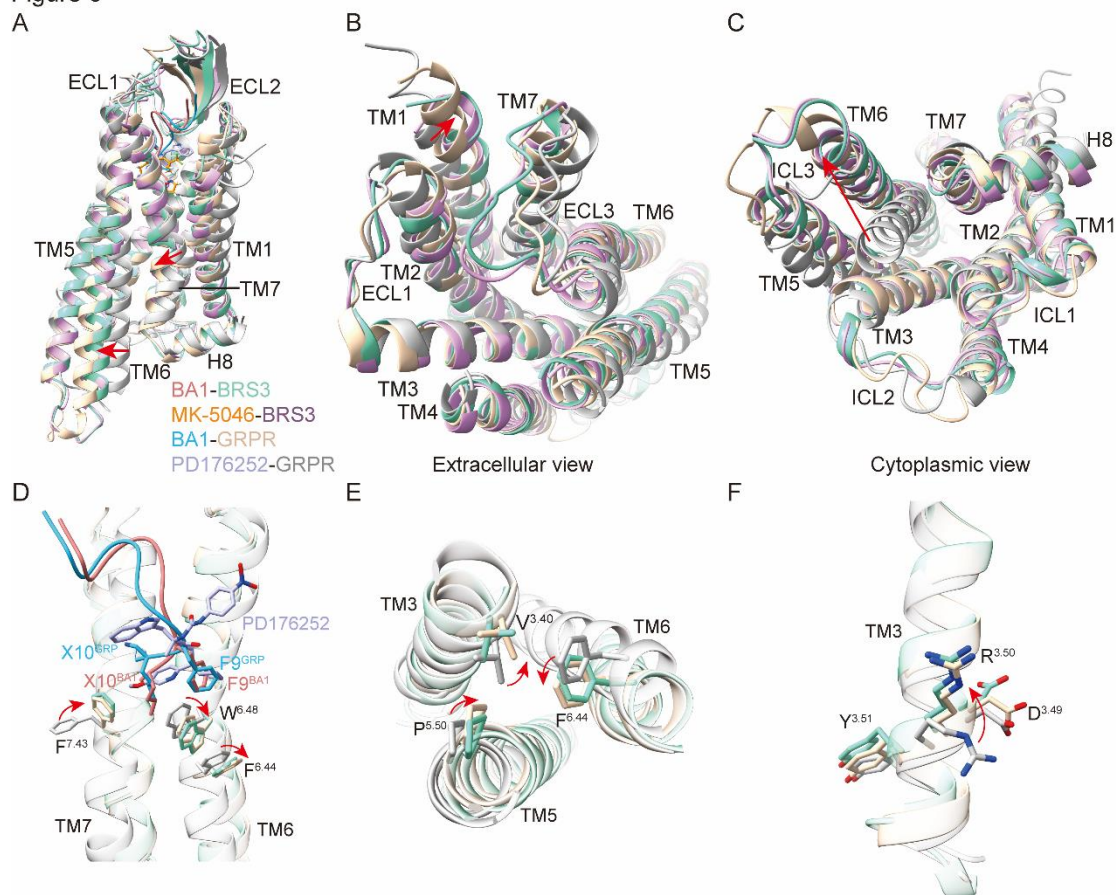


Figure 6. Molecular basis for activation of BRS3.

(A-C) Structural alignment of two active BRS3 in the presence of the ligands BA1 and MK-5046, active BA1-GRPR (PDB code: 7W40) and inactive GRPR (PDB code: 7W41). Side view (A); extracellular view (B); cytoplasmic view (C). The movement directions of TM6 and TM7 in BRS3 relative to inactive GRPR are highlighted as red arrows. Inactive GRPR, active GRPR, active BRS3 in the presence of the ligands BA1 and MK-5046 are colored in gray, burly wood, aquamarine and violet, respectively.

(D-F) Conformational changes of the conserved 'micro-switches' upon receptor activation, including toggle switch (D), PV(I)F (E), and DRY (F) motifs. The conformational changes of residue side chains are shown as red arrows upon receptor activation. The complex structures were aligned by the receptors.

Methods

Constructs

Human BRS3(residues 1-361) was cloned into pFastBac with an N-terminal haemagglutinin (HA) signal peptide followed by thermostabilized apocytochrome b₅₆₂RIL (BRIL)¹. LgBiT was also inserted at the C terminus of the BRS3 using homologous recombination (CloneExpress One Step Cloning Kit, Vazyme). After LgBiT, a tobacco etch virus (TEV) protease cleavage site and a double maltose binding protein (MBP) tag were added to BRS3 constructs for improved expression and purification. The engineered Gα_q construct was generated based on mini-G_{s/q}71², which carries two dominant-negative mutations (G203A and A326S)³ to decrease nucleotide binding affinity and facilitate Gαβγ complex stability. The N-terminal 1-18 amino acids and the α-helical domain of the mini-G_{s/q}71 were substituted with the corresponding sequences of the human Gai1 for binding to the antibody fragments scFv16^{4,5}. Rat Gβ1 was connected with a C-terminal HiBiT by a 15 residues linker. The engineered Gα_q, Gβ1-HiBiT and bovine Gy2 were cloned independently into pFastBac vectors (Invitrogen). The antibody fragment scFv16 was cloned into a modified pFastBac vector containing an N-terminal GP67 secretion signal peptide.

Expression and purification of Nb35

Nanobody-35 (Nb35) with a N-terminal pelB signal peptide and a C-terminal His₆ tag was expressed in the periplasm of Escherichia coli BL21(DE3) bacteria (NEB)⁶. Cultures were grown at 37 °C in Luria-Bertani media containing 50 µg/mL ampicillin to an OD₆₀₀ of 1.0 and induced with 0.1mM IPTG at 28 °C, 180 r.p.m. for another 8 h. Cells were harvested by centrifugation (5316 × g, 30 min) and lysed in ice-cold buffer (20mM HEPES pH 7.4, 500mM NaCl), then centrifuged to remove cell debris. Nb35 was first purified by nickel affinity chromatography, followed by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 prep grade column with a buffer of 20mM HEPES pH 7.4 and 100mM NaCl. Peak fractions were concentrated to 2mg/mL with 15% glycerol and kept frozen at -80 °C for later use. Purified protein quality was assessed by SDS-PAGE.

Expression and purification of complexes

High Five cells (Expression systems) were cultured in ESF921 serum-free medium (Expression Systems) and co-infected with baculoviruses expressing the receptor, $G\alpha_q$, $G\beta_1$, $G\gamma_2$, and scFv16⁷ at a 1:1:1:1:1 ratio for 48 h at 27 °C using Bac-to-Bac baculovirus system. The cell pellets were lysed by dounce homogenization in a buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM TCEP (Sigma-Aldrich), 10% glycerol, and EDTA-free protease inhibitor cocktail (TargetMol). The supernatant was isolated by centrifugation at 65,000 × g for 40 minutes to collect the membranes. The washed membranes were resuspended in buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, agonists (10 μM BA1, and 10 μM MK-5046, Synpeptide and MedChemExpress, respectively), 25 mU/mL apyrase (Sigma-Aldrich), 20ug/mL Nb35, 0.1 mM TCEP and EDTA-free protease inhibitor cocktail, and incubated at 4°C for 8 hours. After incubation, cell membranes were solubilized with 1% (w/v) n-dodecyl-β-d-maltopyranoside (DDM, Anatrace) and 0.2% (w/v) cholesteryl hemisuccinate TRIS salt (CHS, Anatrace) at 4°C for 2 hours. The supernatant was collected by centrifugation at 65,000 × g for 35 minutes and incubated with dextrin resin (Dextrin Beads 6FF, Smart Life Sciences) at 4°C for 5 hours. The resin was washed, eluted, and the protein complexes were further purified by size-exclusion chromatography on a Superdex 200 increase 10/300 GL column (GE Healthcare) in buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM TCEP, agonists (10 μM BA1, 10 μM MK5046, and 1 mM oridonin), 0.00075%(w/v) LMNG, 0.00025% (w/v) GDN and 0.0002% (w/v) CHS. Fractions containing monomeric protein complex were collected and evaluated by SDS-PAGE (Figure S2), and then concentrated by 30-fold for cryo-electron microscopy experiments.

Cryo-EM data collection

Cryo-EM grids were prepared with the Vitrobot Mark IV plunger (FEI) set to 8 °C and 100% humidity. Three-microliter of the BRS3- G_q protein complex in apo state was

applied to the glow discharged copper R1.2/1.3 holey carbon grids. The sample was incubated for 10 s on the grids before blotting for 3.5 s (double-sided, blot force 1) and flash-frozen in liquid ethane immediately. The same condition was used for the sample BA1-BRS3-G_q complex, and MK5046-BRS3-G_q complex.

For BRS3-G_q complex dataset, 3,098 movies were collected on a Titan Krios equipped with a Gatan K3 direct electron detection device at 300 kV with a magnification of 64,000, corresponding to a pixel size 1.08 Å. Image acquisition was performed with EPU Software (FEI Eindhoven, Netherlands). We collected a total of 36 frames accumulating to a total dose of 50 e⁻ Å⁻² over 2.5 s exposure.

For BA1-BRS3-G_q complex dataset, 5,016 movies were collected on a Titan Krios equipped with a Gatan K3 direct electron detection device at 300 kV with a magnification of 105,000, corresponding to a pixel size 0.824 Å. Image acquisition was performed with EPU Software (FEI Eindhoven, Netherlands). We collected a total of 36 frames accumulating to a total dose of 50 e⁻ Å⁻² over 2.5 s exposure.

For MK5046-BRS3-G_q complex dataset, 8,738 movies were collected on a Titan Krios equipped with a Falcon4 direct electron detection device at 300 kV with a magnification of 165,000, corresponding to a pixel size 0.73 Å. Image acquisition was performed with EPU Software (FEI Eindhoven, Netherlands). We collected a total dose of 50 e⁻ Å⁻² over 2.5 s exposure on each EER format movie⁸. Each movie was divided into 36 frames during motion correction.

Cryo-EM image processing

MotionCor2 was used to perform the frame-based motion-correction algorithm to generate drift-corrected micrograph for further processing, and CTFFIND4 provided the estimation of the contrast transfer function (CTF) parameters^{9,10}.

For BRS3-G_q complex dataset, approximately 2,000 particles were manually picked and two-dimensional (2D) classes were calculated and used as references for automatic picking. All subsequent steps including particle picking and extraction, 2D classification, three-dimensional (3D) classification, 3D refinement, CTF refinement, Bayesian polishing, post processing and local resolution estimation were performed using Relion3.0¹¹.

A total of 3,294,877 particles were extracted from the cryo-EM micrographs and followed by two rounds of reference-free 2D classification, yielding 3,270,779 particles after clearance. Three rounds of 3D classification were used to separate out 233,381 particles, which were refined to a structure at 3.93 Å global resolution. After CTF refinement, polishing, and postprocessing, the particles were reconstituted to a 3.29 Å structure. (Figure S3; Table S2).

For BA1-BRS3-G_q complex dataset, the subsequent steps were performed with CryoSPARC¹². Blob-pick was used for the particle picking. 6,245,406 particles were automatic picked and extracted from 5,016 cryo-EM micrographs. After 2 rounds of 2D classification, 799,895 particles were selected from 2,116,883 particles and subjected to generate 5 “good” references and 1,040,145 particles were selected and subjected to generate 5 “bad” references. Two rounds of heterogeneous refinement using “good” and “bad” references yielded 675,060 particles after clearance. We then continued the processing in CryoSPARC. After homogeneous refinement and non-uniform refinement, the particles were reconstituted to a 2.90 Å structure (Figure S4; Table S2).

For datasets of MK-5046-BRS3-G_q complex, the similar strategy was used. After non-uniform refinement, 196,746 particles were reconstituted to 2.93 Å structure, which corresponding to MK-5046-BRS3-G_q complex (Figure S5; Table S2).

Model building

BRS3 structure predicted from Alphafold2 was used as the starting reference models for receptors building¹³. Structures of Gα_q, Gβ, Gγ, the NB35 nanobody, and scFv16 derived from PDB entry 8H0Q¹⁴ were rigid body fit into the density. All models were fitted into the EM density map using UCSF Chimera¹⁵ followed by iterative rounds of manual adjustment and automated rebuilding in COOT¹⁶ and PHENIX¹⁷, respectively. The models were adjusted in ISOLDE¹⁸ followed by refinement in PHENIX. The final model statistics were validated using Comprehensive validation (cryo-EM) in PHENIX¹⁷ and provided in the [Table S2](#). All structural figures were prepared using Chimera¹⁵, Chimera X¹⁹, and PyMOL (Schrödinger, LLC.).

Function assay

AD293 cells (Agilent) were cultured in high glucose DMEM medium (GE healthcare) supplemented with 10% (v/v) fetal bovine serum (FBS, Gemini) and 1% penicillin/streptomycin at 37°C in 5% CO₂ incubator. Inositol phosphate 1 (IP1) production was measured using the IP-One HTRF kit (Cisbio, 621PAPEJ)²⁰. Briefly, cells were seeded onto 12-well plates 16 hours before transfection. Cells were then transiently with different BRS3 constructs using FuGENE HD transfection reagent. After 24 hours, cells were harvested and resuspended at 7×10^5 cells/mL in IP1 stimulation buffer. Cells were then plated onto 384-well assay plates at 4,900 cells/7 µL/well. 7 µL IP1 Stimulation Buffer 2 containing ligand was added and incubated for 1 hour at 37 °C. Intracellular IP1 was measured using the IP-One HTRF kit and an EnVision multiplate reader according to the manufacturer's protocol. The HTRF ratio was converted to a response (%) using: response (%) = ratio of sample/WT×100. Data presented are mean ± S.E.M. of at least three independently biological experiments.

Cell-surface expression assay

Cell-surface expression for each BRS3 mutant was monitored by a fluorescence-activated cell sorting (FACS). The mutants were cloned into pcDNA6.0 vector

(Invitrogen) with a N-terminal FLAG tag. Cells were seeded and transfected as described for the functional assay. After 24 hours of transfection, cells were washed with PBS and detached with 0.2% (w/v) EDTA in PBS. The expressed cells were then incubated with monoclonal anti-FLAG M2-FITC (Sigma-Aldrich) at 1:100 dilution for 15 minutes at 4 °C, followed by a 9-fold excess of PBS. Cells were resuspended and fluorescence intensity was quantified using a BD Accuri C6 flow cytometer (BD Biosciences) with 488 nm excitation and 519 nm emission. FACS data were analyzed with BD Accuri C6 software 1.0.264.21 and normalized to wild-type BRS3.

Computational methods

The simulation systems come from MK-5046-BRS3-G protein complex and BA1-BRS3-G protein complex. G proteins were removed before simulations. Protonation states of residues were determined using Propka3 software²¹. The CHARMM-GUI platform facilitated embedding of these structures into a 75x75 Å POPC lipid bilayer²². The lipid bilayer was then surrounded by a 15 Å aqueous layer. Systems were then adjusted to a 0.15 mol/L NaCl concentration, supplemented with counterions. We applied the CHARMM36m force field for amino acids and lipids, while CHARMM general force field is applied for ligands^{23,24}. The systems underwent a 7-step equilibration process, with gradual minimization and relaxation of constraints, as outlined by CHARMM-GUI. We conducted 3×500 ns independent production runs for each system using pmemd.cuda in Amber20²⁵ under the NPT ensemble at 303.15 K and 1 atm. Long-range electrostatics were managed via the Particle Mesh Ewald method, while short-range electrostatic and van der Waals interactions used a 12 Å cutoff, with a smooth transition between 10 and 12 Å. The hydrophobic network was evaluated using “nativecontact” command in CPPTRAJ. The interacting interface area was calculated using dr_sasa²⁶.

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Figure S1

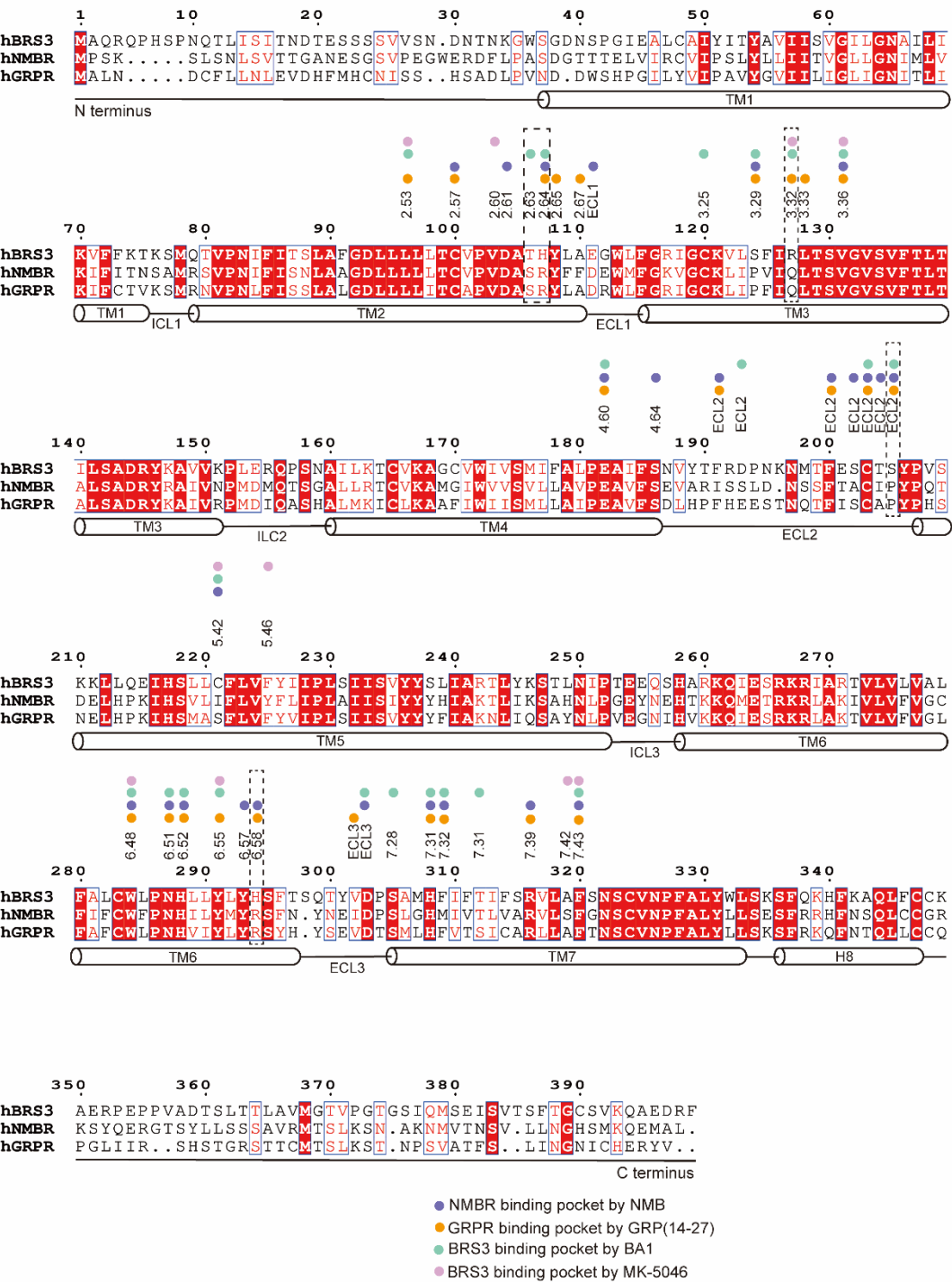


Figure S1. Sequence alignment of BRS3, NMBR, and GRPR. Secondary structure elements are annotated underneath the sequences based on the structure of the BRS3-G_q complex. The ligand-binding pockets of NMB-NMBR, GRP(14-27)-GRPR, BA1-BRS3, and MK-5046-BRS3 is displayed in different colors, purple, orange,

1133 green, and violet, respectively. Additionally, the non-conserved residues including
1134 S^{2.63}, R^{2.64}, Q^{3.32}, P^{45.52}, and R^{6.58}, is circled in dotted box.

Figure S2

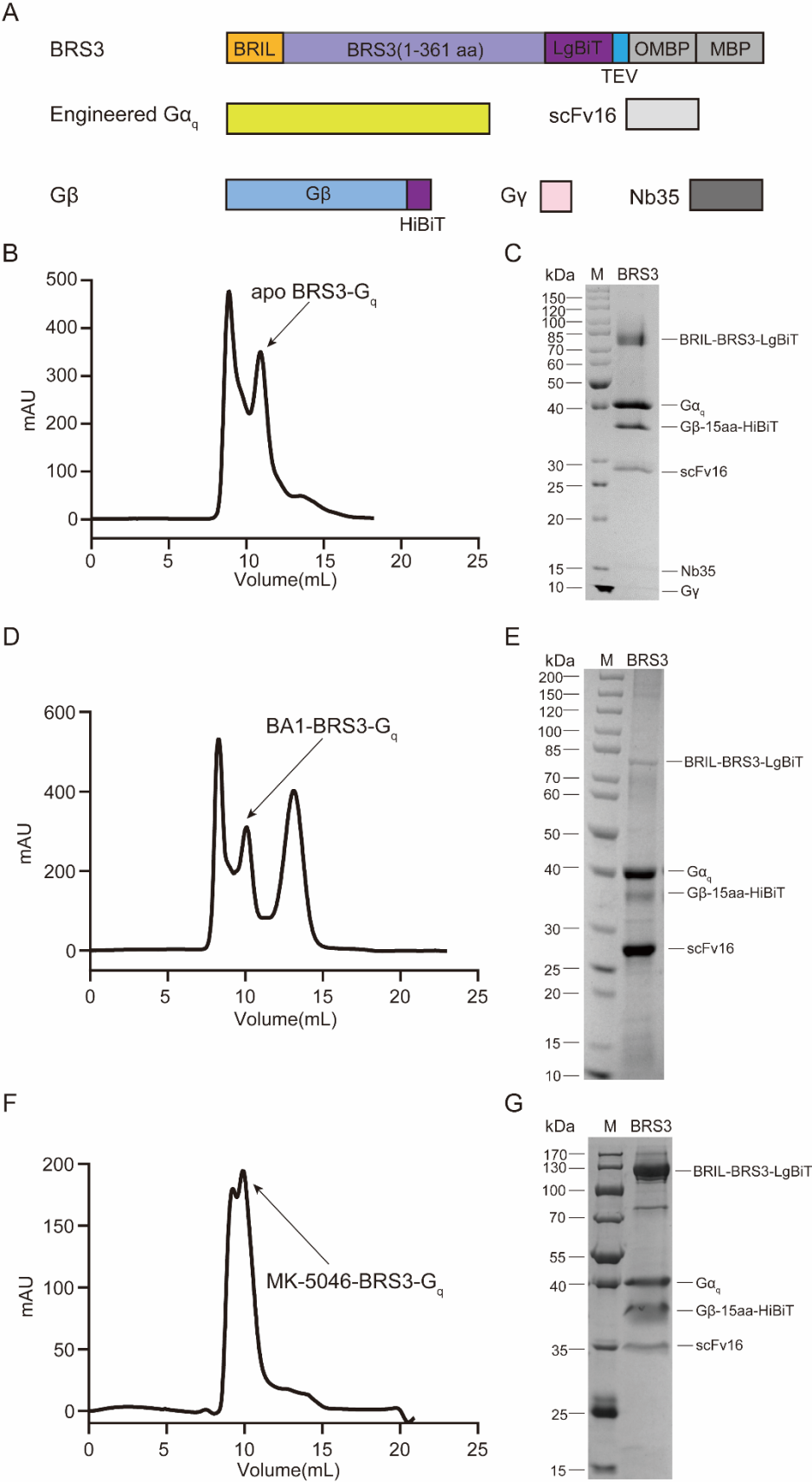


Figure S2. Purification and characterization of BA1-BRS3-G_q complex and MK-5046-BRS3-G_q complex.

(A) Schematic diagram of the protein engineering of BRS3, engineered Gα_q, Gβ, Gγ, scFv16, and Nb35 used in this study.

(B) Gel filtration (Superdex 200 Increase 10/300 column, GE Healthcare) profile of the apo BRS3-G_q complex. The black arrow indicates the sharp peak for apo BRS3-G_q complex.

(C) Coomassie-stained SDS-PAGE analysis of the purified apo BRS3-G_q complex, showing balanced ratios for each subunit.

(D) Gel filtration (Superdex 200 Increase 10/300 column, GE Healthcare) profile of the BA1-BRS3-G_q complex. The black arrow indicates the sharp peak for BA1-BRS3-G_q complex.

(E) Coomassie-stained SDS-PAGE analysis of the purified BA1-BRS3-G_q complex, showing balanced ratios for each subunit.

(F) Gel filtration (Superdex 200 Increase 10/300 column, GE Healthcare) profile of the MK-5046-BRS3-G_q complex. The black arrow indicates the sharp peak for MK-5046-BRS3-G_q complex.

(G) Coomassie-stained SDS-PAGE analysis of the purified MK-5046-BRS3-G_q complex, showing balanced ratios for certain subunits, except for the Gγ subunit.

Figure S3

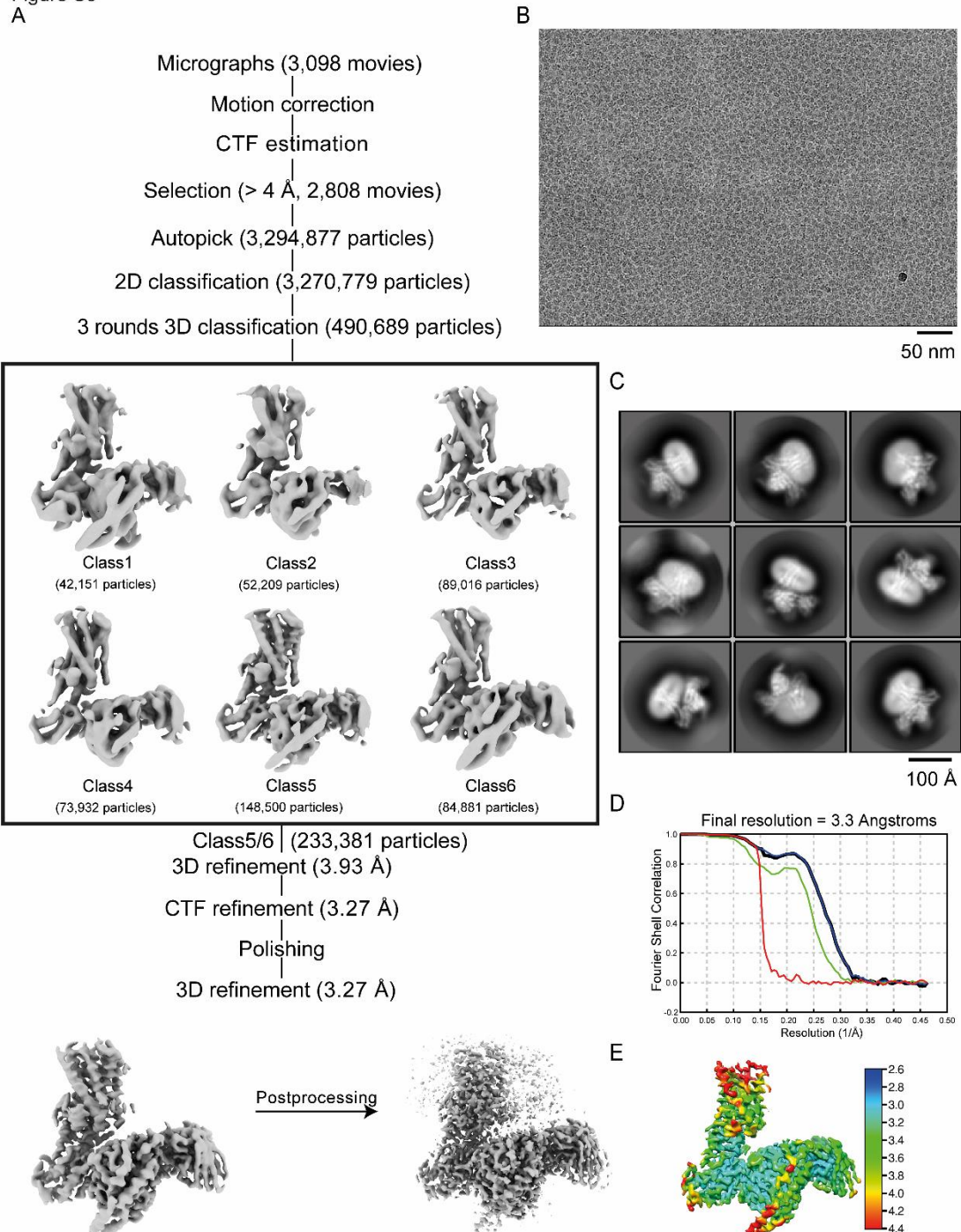


Figure S3. Cryo-EM data processing of apo BRS3-G_q complex.

(A) Flowchart of computational sorting of cryo-EM data.

(B) A representative cryo-EM micrograph of apo BRS3-G_q complex with 50 nm scale bar included as a size reference.

(C) Twelve representative reference-free 2D cryo-EM class averages. Scale bar, 5 nm.

1172 (D) 'Gold-standard' Fourier shell correlation curve of the reconstruction. The resolution
1173 was reported at 3.30 Å using the Fourier shell cutoff at 0.143.

1174 (E) Local resolution map of apo BRS3–G_q complex.

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Figure S4

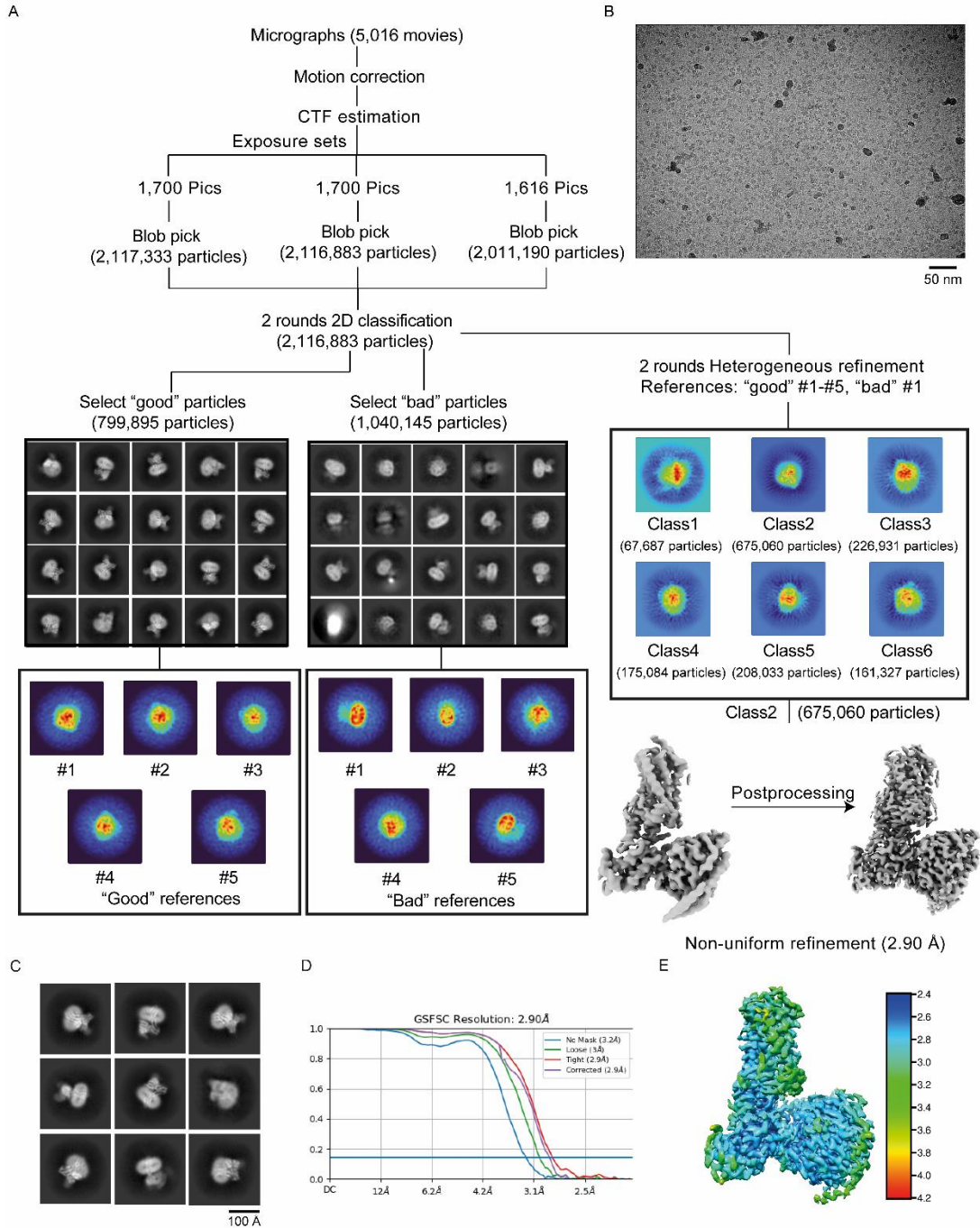


Figure S4. Cryo-EM data processing of BA1-BRS3-G_q complex.

(A) Flowchart of computational sorting of cryo-EM data.

(B) A representative cryo-EM micrograph of BA1-BRS3-G_q complex with 50 nm scale bar included as a size reference.

(C) Twelve representative reference-free 2D cryo-EM class averages. Scale bar, 5 nm.

(D) 'Gold-standard' Fourier shell correlation curve of the reconstruction. The resolution

1205 was reported at 2.90 Å using the Fourier shell cutoff at 0.143.

1206 (E) Local resolution map of BA1-BRS3–G_q complex.

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Figure S5

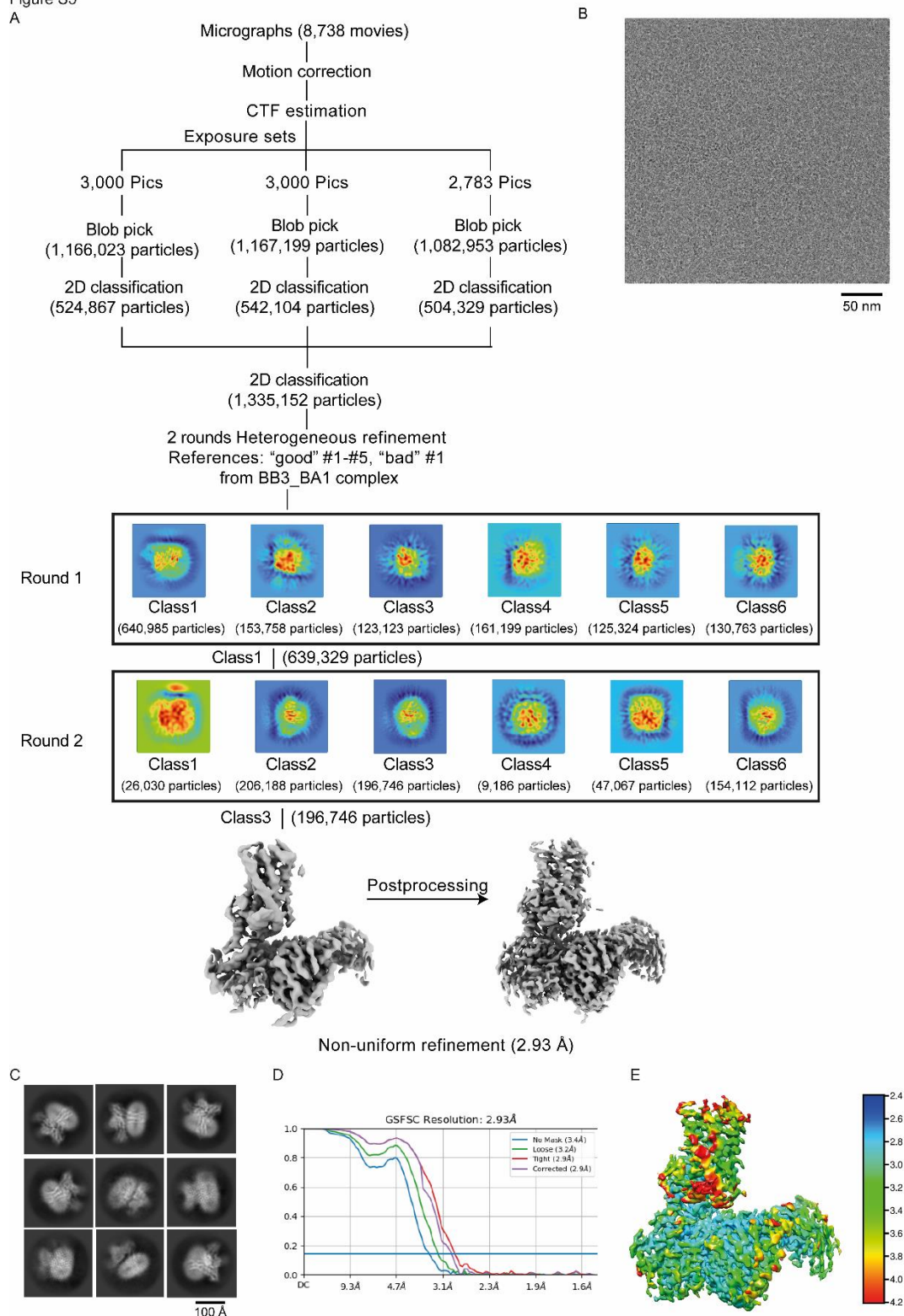


Figure S5. Cryo-EM data processing of MK-5046-BRS3-G_q complex.

(A) Flowchart of computational sorting of cryo-EM data.

(B) A representative cryo-EM micrograph of MK-5046-BRS3-G_q complex with 50 nm

1233 scale bar included as a size reference.

1234 (C) Twelve representative reference-free 2D cryo-EM class averages. Scale bar, 5 nm.

1235 (D) 'Gold-standard' Fourier shell correlation curve of the reconstruction. The resolution

1236 as reported at 2.93 Å using the Fourier shell cutoff at 0.143.

1237 (E) Local resolution map of MK-5046-BRS3-G_q complex.

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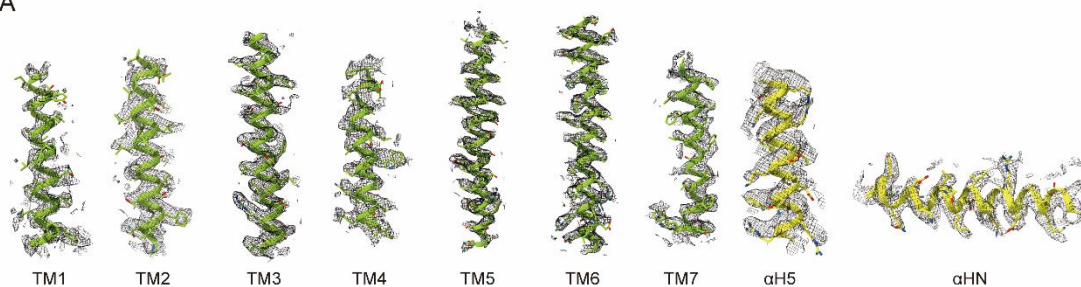
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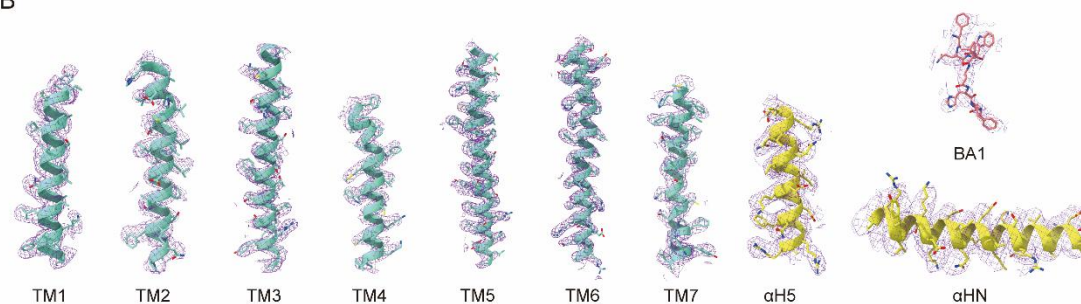
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Figure S6

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B



C

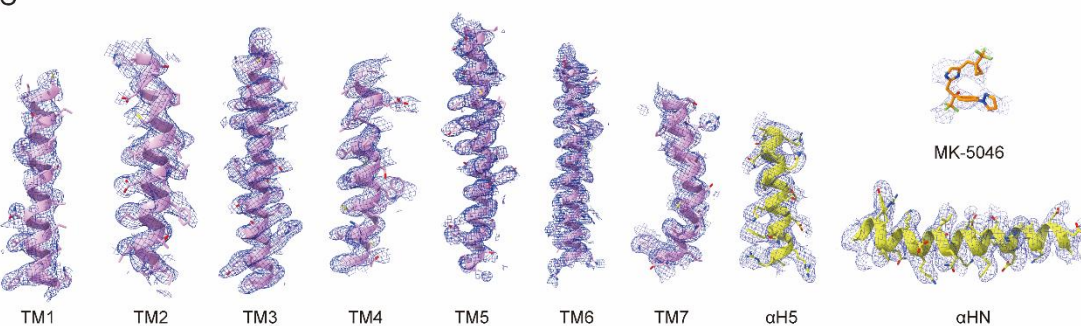
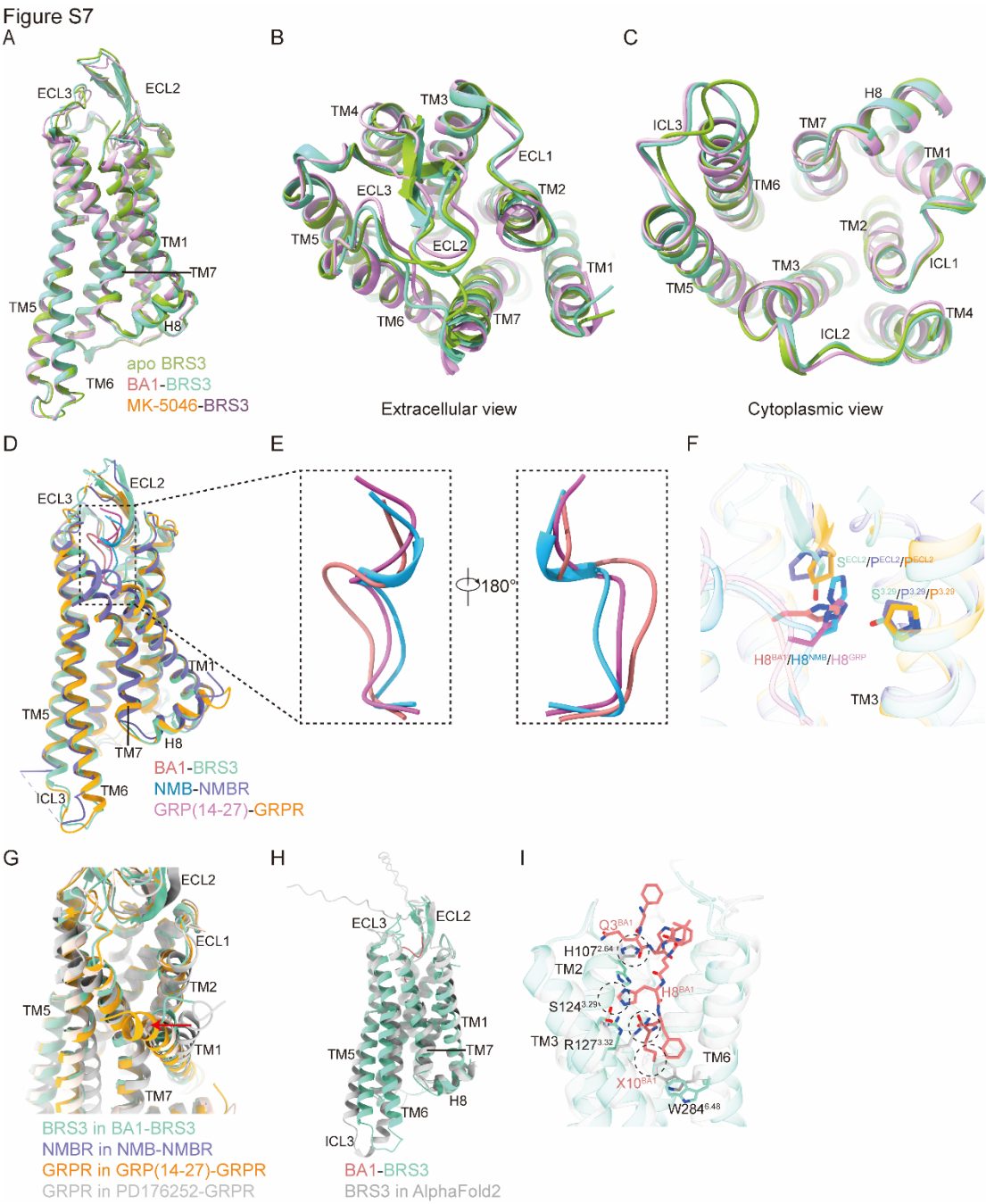


Figure S6. Cryo-EM density maps of TM1-7 in receptors, αH5 and αHN in G_q proteins, and ligands in apo BRS3-G_q complex structure(A), BA1-BRS3-G_q complex structure (B), and MK-5046-BRS3-G_q complex structure (C).



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1277 **Figure S7. Structural comparison of bombesin receptors family.**

1278 (A-C) Different views of the structural superposition of apo-state BRS3 (forest green),

1279 BA1-bound BRS3 (medium aquamarine), MK-5046-bound BRS3 (dark khaki).

1280 (D and E) Side view of the structural superposition of BA1-BRS3, NMB-NMBR, and

1281 GRP(14-27)-GRPR(D), and three ligands(E).

(F) The H8^B residue forms hydrogen bonds with S124^{3.29} and S205^{ECL2}, differing from its analogs in NMB and GRP(14-27).

(G) The structural superposition of BRS3 in BA1-BRS3, NMBR in NMB-NMBR, GRPR in GRP(14-27)-GRPR, and GRPR in PD176252-GRPR.

(H and I) The structural comparison of BRS3 in BA1-BRS3 and AlphaFold2 website(H) and the clashes between BA1 and OBP in the AlphaFold2 BRS3 structure(I).

Figure S8

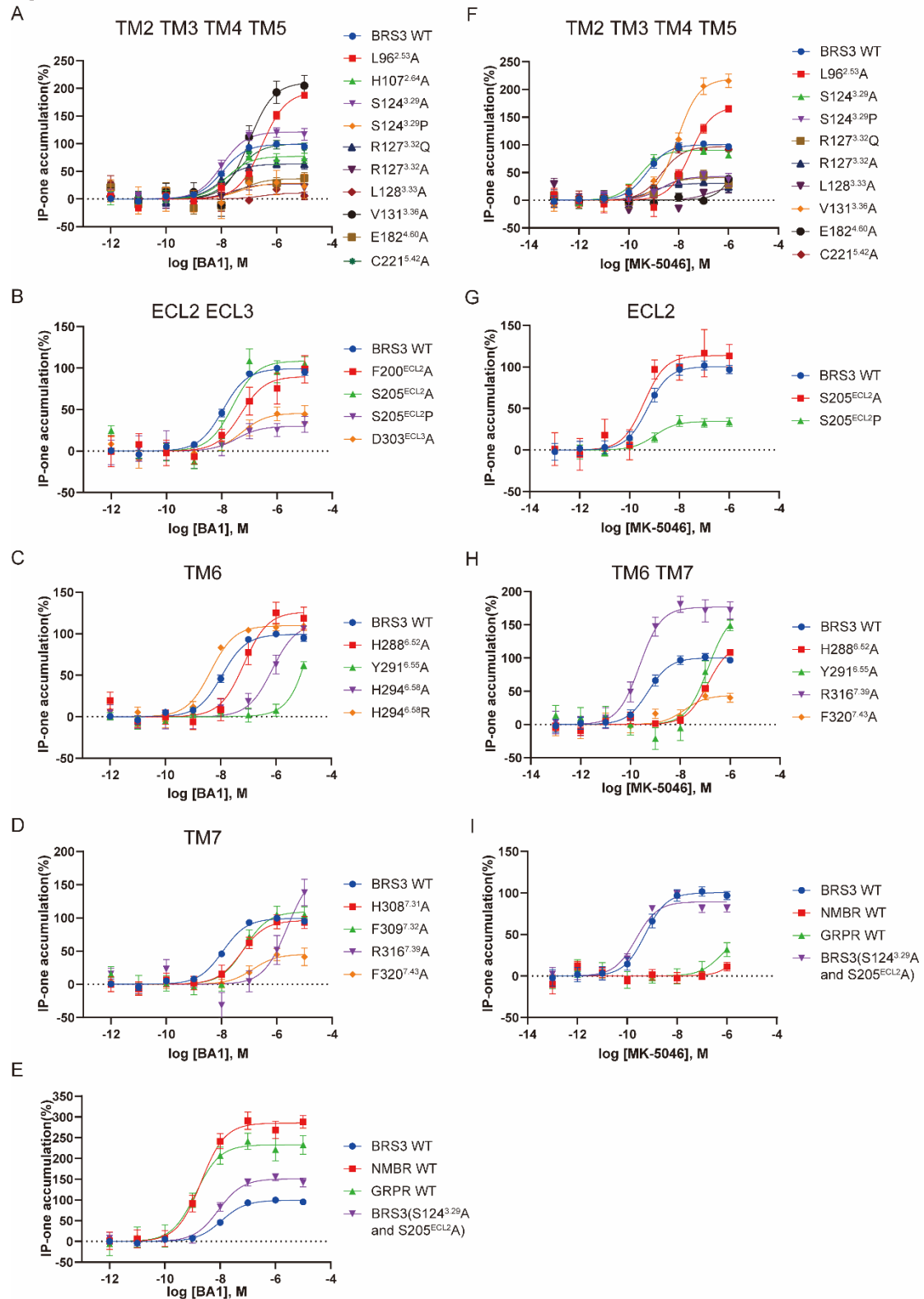


Figure S8. IP1 response curves of BA1 and MK-5046. BRS3 mutations on agonists BA1 (A-E) and MK-5046 (F-I) induced signaling effects. Dose-response curves for

ligands were performed in transiently transfected AD293 cells. The results are expressed as percentage of the maximal effect observed in BRS3 wild type. Wild-type and mutated BRS3 data are shown by different colors as figures. Each point represents the mean \pm S.E.M. of 3 to 7 independent experiments with triplicate determinations.

Figure S9

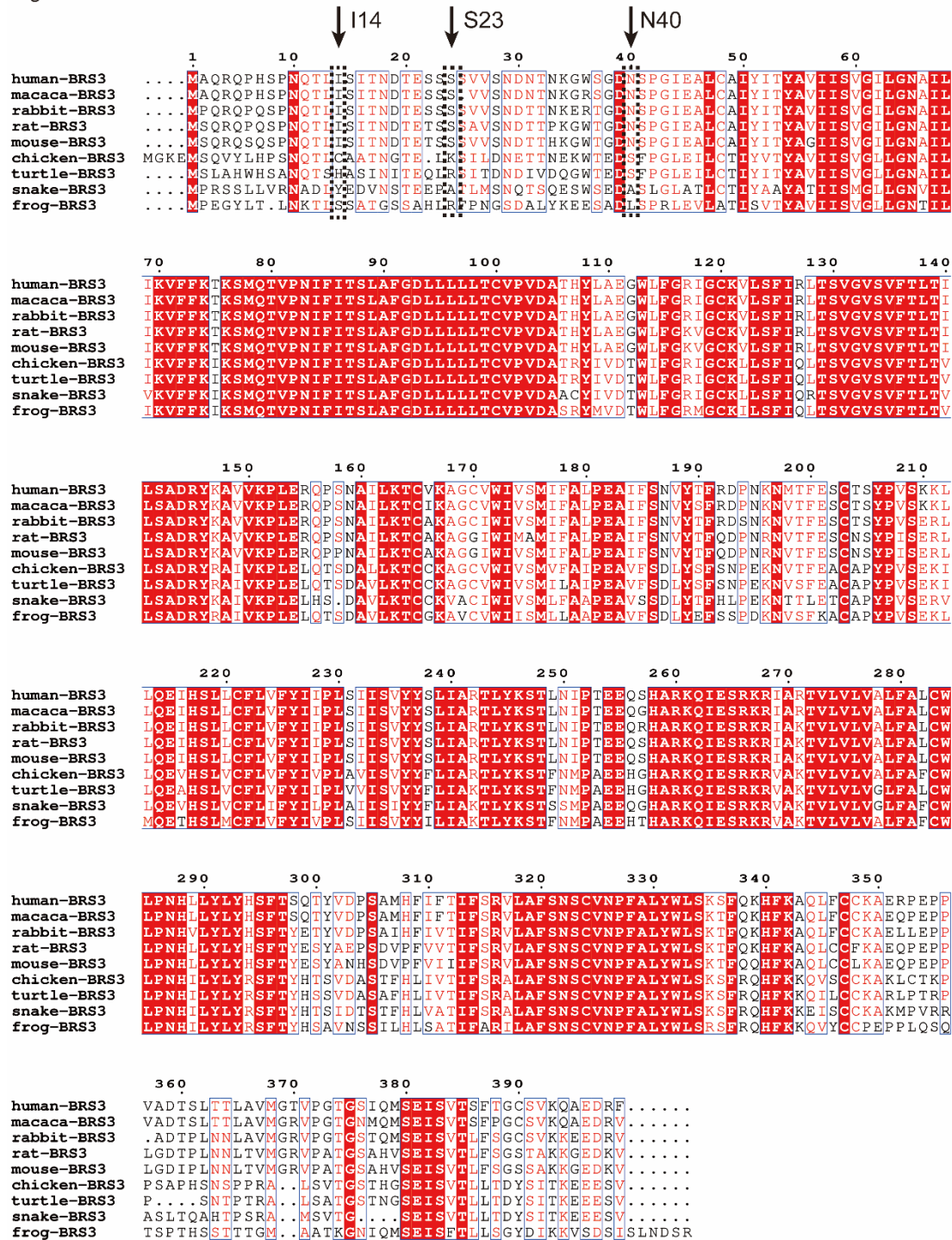


Figure S9. Sequence alignment of BRS3 of different species including placental mammals (human, macaca mulatta, rabbit, rat, and mouse) and non-placental vertebrates (chicken, turtle, snake, and frog).

Table S1. The affinity(pK_i) data of Bn-related ligands for human bombesin receptor family.

	pK_i^*			
	NMB	GRP(14-27)	BA1	MK-5046
hNMBR	8.1-10.3	6.8-6.9	7.8-9.7	<5.0
hGRPR	7.7-7.8	9.7-9.8	7.8-8.0	<5.0
hBRS3	5.7	<5.0	8.1-8.4	7.7-8.4

*The data are from the website "<https://www.guidetopharmacology.org/>".

Table S2. Cryo-EM data collection, model refinement and validation statistics.

	apo BRS3-G _q - scFv16 complex	BA1-bound BRS3-G _q complex	MK-5046-bound BRS3-G _q - scFv16-Nb35 complex
Data collection and processing			
Detector	K3	K3	Falcon 4
Magnification	64,000	105,000	165,000
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å ²)	50	50	50
Defocus range (μm)	-1.0~3.0	-1.0~3.0	-1.0~3.0
Pixel size (Å)	1.08	0.824	0.73
Symmetry imposed	C1	C1	C1
Initial particle projections (no.)	3,294,877	6,245,406	3,416,175
Final particle projections (no.)	233,381	675,060	196,746
Map resolution (Å)	3.3	2.90	2.93
Map resolution range (Å)	2.60-4.40	2.40-4.20	2.40-4.20
FSC threshold	0.143	0.143	0.143
Model Refinement			
Refinement package	PHENIX-1.17.1-3660	PHENIX-1.17.1-3660	PHENIX-1.17.1-3660
Real or reciprocal space	Real space	Real space	Real space
Model-Map CC (mask)	0.43	0.63	0.62
Model resolution (Å)	3.30	2.87	3.70
FSC threshold	0.143	0.143	0.5
B factors (Å ² , min/max/mean value)			
Protein residues	22.81/126.95/65.54	20.00/126.95/64.51	22.81/155.34/71.34
Ligands	-/-	20.00/20.00/20.00	20.00/20.00/20.00
Model composition			
Non-hydrogen atoms	9,067	7,380	10,071
Protein residues	1,153	930	1,281
R.m.s. deviations			
Bond lengths (Å)	0.001	0.003	0.003
Bond angles (°)	0.386	0.649	0.924
Validation			
MolProbity score	1.24	1.26	1.19
Clashscore	4.70	5.01	4.09
Rotamer outliers (%)	0.10	0.25	0.09
Ramachandran plot			
Favored (%)	98.59	98.47	98.42
Allowed (%)	1.41	1.53	1.58
Disallowed (%)	0.00	0.00	0.00
Data availability			
EMDB entry	EMD-38927	EMD-38928	EMD-38929
PDB entry	8Y51	8Y52	8Y53

Table S3. Sequence of Bn-related peptides used or mentioned in this study.

Peptide name	N-Terminus	position relative to Bn													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Endogenous															
Bn(Bombesin)		pE	Q	R	L	G	N	Q	W	A	V	G	H	L	M-NH ₂
GRP27(GRP(1–27))	VPLPAGGGTVLTK	M	Y	P	R	G	N	H	W	A	V	G	H	L	M-NH ₂
GRP (14–27)a(GRP (14–27))		M	Y	P	R	G	N	H	W	A	V	G	H	L	M-NH ₂
GRP (14–27)b(GRP (14–27))		M	Y	P	R	G	N	H	W	A	V	G	H	L	M
NMB(Neuromedin B)						G	N	L	W	A	T	G	H	F	M-NH ₂
NMB30(Neuromedin B (1–30))	LSWDLPEPRSRASKIR	V	H	R	R	G	N	L	W	A	T	G	H	F	M-NH ₂
NMC(Neuromedin C)						G	N	H	W	A	V	G	H	L	M-NH ₂
Exogenous															
BA1([D-Phe ⁶ ,β-Ala ¹¹ , Phe ¹³ ,Nle ¹⁴]bombesin-(6–14))							^D F	Q	W	A	V	^β A	H	F	X
		1	2	3	4	5	6	7	8	9	10				
		position relative to NMB or NMC													

Table S4. Ligand binding affinities and expression levels of WT and mutated BRS3. The wild type (WT) and mutants of BRS3 discussed in this manuscript were individually analyzed. The affinities are derived from at least 3 independent experiments using IP1 function assay. The expression level of mutant BRS3 were normalized to wild-type BRS3 as 100%, respectively. Each data point represents mean \pm standard error of the mean (S.E.M.). All data were analyzed by two-sided Student's t test. *P<0.05, **P<0.01, ***P<0.001 vs. WT. Source data are available online. Definitions: NA – not applicable; NT, not tested.

Residue Number	BRS3 mutants	Expression (% of WT)	BA1 pEC ₅₀ ±S.E.M.	BA1 ±S.E.M.(%WT)	E _{max} MK-5046 pEC ₅₀ ±S.E.M.	MK-5046 E _{max} ±S.E.M.(%WT)
-	WT	100±0.60	-7.81±0.10	98.95±0.93	-9.3±0.08	99.61±5.08
2.53	L96A	56.14±0.57	-6.53±0.06***	194.35±2.50***	-7.51±0.08**	169.55±5.24***
2.64	H107A	49.66±2.25	-7.82±0.08	77.26±15.79*	NT	NT
3.29	S124A	88.27±0.52	-7.98±0.03	121.41±17.26*	-9.52±0.12	99.31±31.63
3.29	S124P	46.96±0.90	-7.83±0.20	3.33±5.12***	-8.45±0.47	47.27±11.52***
3.32	R127A	78.5±1.49	-7.5±0.23	27.57±18.50***	-9.12±0.25	30.09±4.74***
3.32	R127Q	88.55±1.84	-8.06±0.11	63.09±13.39***	-9.06±0.16	53.85±10.57**
3.33	L128A	69.71±1.14	NA	NA	NA	NA
3.36	V131A	49.89±1.91	-6.98±0.06**	211.09±33.36***	-8.04±0.15	220.25±26.06***
4.60	E182A	63.43±2.10	-7.13±0.06**	74.21±5.15***	-4.93±3.56*	NA
ECL2	F200A	51.62±1.89	-6.98±0.79	110.6±38.69	NT	NT
ECL2	S205A	61.14±0.37	-7.59±0.10	109.11±18.74	-9.46±0.14	100.42±6.74
ECL2	S205P	119.29±0.27	-7.33±0.12*	30.57±14.93***	-9.06±0.34	34.47±8.97***
5.42	C221A	40.62±0.58	-7.38±0.18	129.13±23.94*	-8.67±0.21	96.99±4.55
6.52	H288A	34.09±0.29	-7.14±0.15**	127.01±18.88*	-6.9±0.07***	NA
6.55	Y291A	93.13±0.58	-5.22±0.12***	NA	-6.77±0.21***	NA
6.58	H294A	94.44±1.20	-6.15±0.16***	114.14±13.68*	NT	NT
6.58	H294R	76.83±1.04	-8.34±0.11*	110.1±7.27*	NT	NT
ECL3	D303A	60.11±1.58	-7.34±0.28	45.94±16.74***	NT	NT
7.31	H308A	82.8±0.17	-7.31±0.27*	97.07±17.95	NT	NT
7.32	F309A	98.74±2.23	-7.17±0.06**	109.52±24.06	NT	NT
7.39	R316A	33.25±0.30	-5.88±0.04***	NA	-9.62±0.04	176.84±25.63***
7.43	F320A	43.75±1.26	-6.87±0.07***	45.31±5.73***	-8.49±0.58	40.28±5.61***
3.29, ECL2	S124A, S205A	14.41±0.08	-8.06±0.04	151.15±13.19***	-9.65±0.11	89.3±3.87
	NMBR-WT	73.17±2.73	-8.66±0.11**	285.63±35.44***	NA	NA
	GRPR-WT	42.71±0.73	-8.85±0.10***	200.2±37.77***	NA	NA

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Table S5. Sequence alignment of the key residues in sodium site, DRY motif, PV(I)F motif, toggle switch and NPxxY motif, as well as residues involved in disulfide bond formation in bombesin receptors.

	Sodium site		Disulfide bond			DRY motif			PV(I)F motif			toggle switch			NPxxY motif		
Residue position	2.50	3.39	3.25	4.64	5.34	3.49	3.50	3.51	5.50	3.40	6.44	6.44	6.48	7.43	7.49	7.50	7.54
NMBR	D	S	C	T	H	D	R	Y	P	V	F	F	W	F	N	P	Y
GRPR	D	S	C	S	H	D	R	Y	P	V	F	F	W	F	N	P	Y
BRS3	D	S	C	S	L	D	R	Y	P	V	F	F	W	F	N	P	Y
Class A conserved	D	S	C	x	x	D	R	Y	P	V(I)	F	F	W	F/Y	N	P	Y